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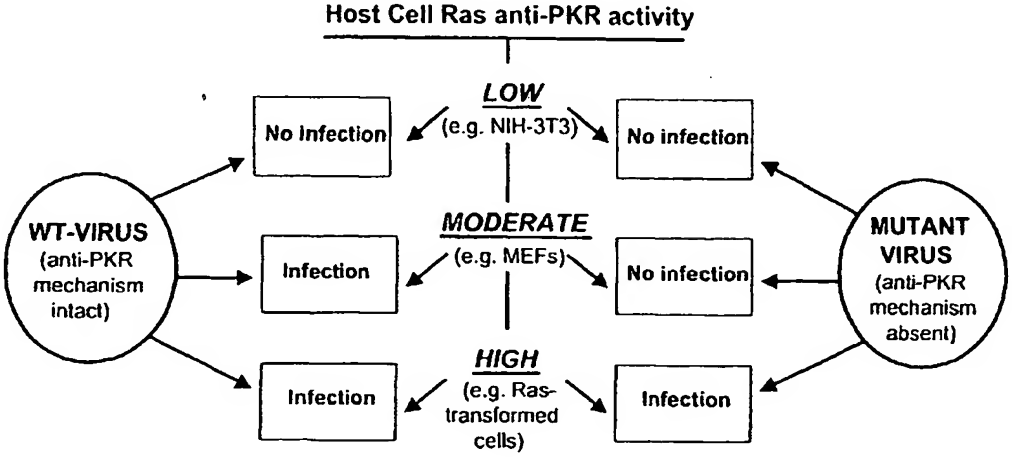
(54) Title: ENGINEERING ONCOLYTIC VIRUSES



(57) Abstract: A method for engineering oncolytic viruses is described, which method comprises alteration or deletion of a viral anti-PKR activity.

WO 03/008586 A2

ENGINEERING ONCOLYTIC VIRUSES

FIELD OF THE INVENTION

The present invention relates to a method for engineering viruses to be anti-cancer agents.

BACKGROUND OF INVENTION

Considerable effort has recently been directed at the potential use of viruses, including wild-type viral strain and attenuated mutants of herpes viruses, as anti-cancer agents by direct lysis of cancer cells while sparing normal cells [1-3]. Attenuated herpes simplex viruses (HSV's) have been shown to selectively infect cancer cells while sparing normal cells. One such mutant of HSV-1 (designated R3616), which has the neurovirulence gene $\gamma_1$34.5 deleted from both loci, is effective in the treatment of experimental brain tumors [2, 4,5]. Derivatives of this mutant (e.g. G207) are effective against some human tumors implanted in mice [6-10], and are currently being used in clinical trials. Viral strain has also been found to be effective in the treatment of cancers [11].

Overactivation of the proto-oncogene *ras* and its signaling pathway is believed to contribute to approximately 30% of all human tumors [12,13]. The role that Ras plays in the pathogenesis of human tumors is specific to the type of tumor. Activating mutations in *ras* are found in most types of human malignancies, and are highly represented in pancreatic cancer (80%), sporadic colorectal carcinomas (40-50%), human lung adenocarcinomas (15-24%), thyroid tumors (50%) and myeloid leukemia (30%) [14-16].

Double-stranded RNA-activated protein kinase (PKR) in its phosphorylated state has been hypothesized to lead to an inhibition of viral RNA translation and thereby make cells non-permissive to viral infection. Activation of *ras* is correlated with a decreased level of phosphorylated-PKR in cells that are infected with wild-type viral strain [11]. The permissiveness of cells expressing activated *ras* to infection by wild-type viral strain, is found to be related to the decreased level of PKR-phosphorylation in these cells [11].

The mechanism of oncolysis using attenuated HSV-1 mutants has yet to be elucidated. In view of the potential use of HSV as an anti-cancer therapeutic, it is

imperative that the mechanism of HSV oncolysis be defined in relatively precise terms. By understanding how these attenuated HSV-1 viruses mediate oncolysis, we can transmit this knowledge to other viruses that, as of yet, have not been shown to have potential in anti-cancer therapeutics.

SUMMARY OF THE INVENTION

The applicants have discovered that mammalian cells that are transformed with oncogenes that activate the Ras signaling pathway are more permissive to HSV-1 infection than untransformed cells, and this permissiveness is linked to the inhibition of virus-induced activation (phosphorylation) of double-stranded RNA-activated protein kinase (PKR). By inhibiting components of the Ras pathway, the applicants were able to show that PKR phosphorylation was restored and viral replication was inhibited. In addition to HSV-1 and other herpes viruses, other viruses such as viral strain and influenza viruses also utilize the Ras signaling pathway. Cells which have an activated Ras pathway may be more permissive to all such viruses.

It is thus one object of this invention to provide a method for engineering viruses to be anti-cancer agents based upon the alteration or elimination of an inherent viral anti-PKR activity.

In one embodiment this method comprises the following steps, which are used for viruses that are not known to possess an inherent anti-PKR activity:

(A) determining whether a viral strain has inherent viral anti-PKR activity;

(B) identifying the viral gene or at least one of the viral genes responsible for the viral anti-PKR activity; and

(C) altering at least one of the viral gene or genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity, relative to the viral strain, is created.

In another embodiment this method comprises the following steps, which are used for viruses that are known to possess an inherent anti-PKR activity:

(A) identifying the viral gene or at least one of the viral genes responsible for the viral anti-PKR activity, and

(B) altering the viral gene or genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity, relative to the viral strain, is created.

In yet another embodiment this method comprises the following step, which is used for viruses for which the inherent anti-PKR activity is known to result from a particular viral gene or genes:

(A) altering the viral gene or genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity, relative to the viral strain, is created.

Any of the above methods may further comprise the steps of:

(A) testing the mutant viral strain to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to infection by the mutant viral strain than are normal cells, and

(B) selecting the mutant viral strain as the viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the mutant viral strain than are normal cells.

In another aspect, this invention is a method of identifying a viral strain useful as an anti-cancer agent, comprising the steps of:

(A) determining whether the viral strain has a low or non-existent anti-PKR activity,

(B) testing the viral strain in culture, if the viral strain has a low or non-existent anti-PKR activity, to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to the viral strain than are normal cells, and

(C) identifying the viral strain as a viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the viral strain than are normal cells.

The viral strain may be a wild-type strain, a genetically engineered strain, or mutant strain. In another aspect, this invention is the use of the mutant viral strains thus created or identified, as anti-cancer agents or in medicaments for treating cancer. In another aspect, this invention is a method for using the mutant viral strains thus created or identified, as anti-cancer agents.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: A comparison, by immunoflourescence, of host cell permissiveness to HSV-1 infection of NIH-3T3 cells and NIH-3T3 cells that are transformed with various oncogenes.

Figure 2: A comparison, by immunoblotting of viral proteins, of permissiveness to HSV-1 infection of NIH-3T3 cells and NIH-3T3 cells that are transformed with various oncogenes.

Figure 3: A comparison, by plaque titration, of HSV-1 virus yield from HSV-1 infected NIH-3T3 cells and NIH-3T3 cells that are transformed with various oncogenes. The upper panel (A) shows the results using a MOI of 0.5 PFU/cell, whereas the lower panel (B) shows the results using a MOI of 5.0 PFU/cell.

Figure 4: Panel A shows a comparison, by immunoblotting of viral proteins, of the effect of FTI-1, an ERK pathway inhibitor (PD98059) and a p38 pathway inhibitor (SB203580) on the ability of HSV-1 to infect *H-ras* transformed cell lines. Panel B shows the effect of FTI-1 and PD98059 on Erk42/44 phosphorylation, and the effect of SB203580 on ATF2 phosphorylation as an activity control for these chemicals.

Figure 5: Panel A shows the effect of three different inhibitors, FPTI-1, FPTI-2 and FTI-4 on HSV-1 viral protein synthesis, as compared to control (*H-ras* transformed) cells that have not been exposed to inhibitors. Panel B shows the effect of 100 μM FTI-1 on HSV-1-infected A549 cells (human lung carcinoma, a standard cell line for HSV growth), using immunofluorescence.

Figure 6: HSV-1 virus yield from *H-ras* transformed cell lines in the presence of FTI- I in two different concentrations, PD98059 and SB203580 in two different concentrations.

Figure 7: The left panel shows a comparison, by immunoblotting of viral proteins, of the effect of Wortmannin, an inhibitor of PI3-kinase on the ability of HSV-1 to infect *H-ras* transformed cell lines, and the effect of Wortmannin on Akt phosphorylation. The right panel shows the ability of HSV-1 to infect NIH-3T3 cells that express Ras effector domain mutants. The mutant cell lines are V12C40 (C40), V12G37(G37) and V12S35(S35). PDCR represents NIH-3T3 cells which have been transfected with control vector and exert no Ras overactivity.

Figure 8: Panel A shows quantitative RT-PCR of early, middle and late HSV-1 gene expression in HSV-1 infected NIH 3T3 cells and *H-ras* transformed cells. Panel B shows a comparison, by immunoblotting, of the level of ICP27, ICP8 and gC, in HSV-1 infected NIH-3T3 cells and *H-ras* transformed NIH-3T3 cells.

Figure 9: Panel A shows an immunoblot comparison showing the early detection of HSV-1 infection in *THC-11* and *H-ras* transformed cell lines as compared to A549 cell line. Panel B shows that infection of H-ras cells by HSV-1 can be detected using immunofluorescence with anti-gC antibody as early as 8 hours after the cells have been exposed to the virus.

Figure 10: Panel A shows a comparison, by immunoblotting, of the phosphorylation state of PKR and eIF-2α in HSV-1 infected and uninfected cell lines (NIH-3T3 and oncogene-transformed NIH-3T3 cell lines). Panel B shows a comparison, by immunoblotting, of the phosphorylation state of PKR in HSV-1 infected and uninfected cell lines (NIH-3T3 and oncogene-transformed NIH-3T3 cell lines) after exposure to FTI-1 and PD98059 (left panel). A comparison, by immunoblotting, of phosphorylation state of PKR in HSV-1 infected NIH-3T3 cells and MEF cells (right panel).

Figure 11: A comparison, by immunoblotting, of the level of viral protein synthesis in NIH-3T3 cells and oncogene-transformed cell lines, after infection with R3616.

Figure 12: A comparison, by immunoblotting, of the level of viral protein synthesis after infection of $PKR^{+/+}$ and $PKR^{-/-}$ fibroblasts with HSV-1 and R3616.

Figure 13: A comparison, by immunoblotting, of the level of viral protein synthesis after infection of $PKR^{+/+}$ and $PKR^{-/-}$ fibroblasts with HSV-1 and exposure to FTI-1.

Figure 14: Model demonstrating how the host cell and viral anti-PKR mechanisms can influence the infection of normal and transformed cells by wild-type and mutant HSV-1 virus.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise indicated, all terms used herein have the same meaning as is commonly understood by one skilled in the art of the present invention. Practitioners are particularly directed to Maniatis *et al.,* in Molecular Cloning (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory), and Ausubel *et al.,* in Current Protocols in Molecular Biology (John Wiley and Sons, Inc.), the contents of which are incorporated herein by reference, for terms of the art.

As used herein the following terms have the following meanings:

"anti-cancer agent" means an agent which kills cancer cells or interferes with the viability or replication of cancer cells. Ideally, such an agent has a reduced or no effect on normal or non-cancerous cells.

"anti-PKR activity" includes any activity which has the consequence of opposing, countering or acting contrary to, the PKR system, or the effect of the PKR system. The anti-PKR activity may originate from a cellular or viral element and can be directed against PKR itself, or elements upstream or downstream of PKR, such as, for example eIF-2α.

"cancer" or **"cancerous"** as used herein are synonymous with tumor or tumorous and cancer or neoplastic, and includes cultured cells of cancerous, tumorous or neoplastic tissues, in the appropriate context.

"herpes or **herpes virus"** includes herpes simplex virus type 1 (HSV-1); herpes simplex virus type 2 (HSV-2); varicella-zoster virus (VZV); cytomegalovirus (CMV); Epstein-Barr virus (EBV) and various other human herpes viruses (HHV) such as HHV-6, HHV-7 and HHV-8.

"HSV" means herpes simplex virus, and includes both type-1 and type-2.

"HSV-1 infected" refers to cells that have been exposed to HSV-1 virus, and does not incorporate any reference as to whether the cells are permissive or non-permissive to the

virus. In the appropriate context, HSV-1 infected may mean cells that are exhibiting signs of active infection.

"**oncogene-transformed cell lines**" means cell lines that are transformed with an oncogene.

"**oncolytic**" refers to an agent which kills cancerous cells.

"**Ras pathway**" includes signal transduction pathways that are downstream of receptor tyrosine kinases (RTK's) such as epidermal growth factor receptors or non-receptor kinases (nRTK's) such as the *src* family kinases, which can lead to the activation of Ras and its downstream elements. As used herein, "Ras pathway" includes other biochemical pathways which lead or can lead to the activation of Ras, or its upstream or downstream elements (e.g. through activation of any G proteins, RAL, RAP, PI3 kinase, PKC, Calcium, FAK etc.). Pathways downstream of Ras which are included in this definition include the MAPK cascade consisting of Raf isoforms, MEK1/2, and ERK1/2.

"**non-permissive cells**" refers to cells that do not support virus growth as demonstrated by the substantial lack of cytopathic effects, viral protein synthesis or virus output after exposure to a virus.

"**permissive cells**" refers to cells that support virus growth as demonstrated by the induction of substantial cytopathic effects, substantial viral protein synthesis or virus output after exposure to a virus.

As described herein, the applicants have discovered that HSV-1 exploits an activated tyrosine receptor kinase/Ras pathway for infection. NIH-3T3 cells, which are non-permissive to HSV-1 infection, become permissive when transformed by the oncogenes *v-erb B*, *Sos*, or *H-Ras*. These oncogenes are all activators of the Ras signaling pathway. Permissiveness of these cells to HSV-1 infection is defined by the induction of cytopathic effects, enhanced viral protein synthesis, and/or production of progeny HSV-1 virus. The applicants have demonstrated that cells non-permissive to HSV-1 infection inhibit viral replication at the protein translational level by phosphorylated PKR. Cells permissive to HSV-1 infection have an activated Ras pathway that dephosphorylates or prevents the phosphorylation of PKR (or its down stream elements like eIF2α), which allows viral protein translation to proceed.

The applicants have shown that the farnesyl transferase inhibitors FTI-1 and FPTI-II effectively block HSV-1 infection in *H-ras* transformed NIH-3T3 cells, which otherwise are permissive to HSV-1 infection. Posttranslational farnesylation of Ras is necessary for association of Ras with the plasma membrane, and is known to be important for the initiation of downstream events, including distinct MAPK cascades, (eg. ERK, JNK) [17-21]. The enzyme farnesyl transferase covalently links a farnesyl group (15 carbon isoprenoid) to a cysteine residue located in the carboxy terminal CAAX motif of Ras, allowing the latter to be anchored to the plasma membrane. Farnesyl transferase inhibitors have been developed as potential anti-cancer agents that block farnesylation and thus inhibit the function of oncogenic Ras. Without being limited to a theory, it appears that the inhibition of HSV-1 replication by farnesyl transferase inhibitors means that HSV-1 infection requires an activated Ras pathway of the host cell.

Extracellular signals received by cell surface receptors are transformed into intracellular instructions that coordinate the appropriate cellular responses [20]. Nearly all cells use one or more MAPK (mitogen-activated protein kinase) cascades to accomplish this. The ERK pathway (extracellular signal regulated kinase) is one such cascade, which acts downstream of Ras to regulate cellular growth [20-22]. Ras regulates the activity of Raf, a serine-threonine kinase in this pathway. Raf activates MEK1/2 [MAPK/ERK kinase], which activates ERK1/2, one of the latter members in a pathway that plays a role in cellular proliferation and differentiation.

The applicants have further shown that MEK1/2 activity is required for HSV-1 infection. PD98059 [24], an inhibitor of MEK1/2, partially blocked HSV-1 infection in *H-ras* transformed NIH-3T3 cells. Combined, these results show that the ERK pathway is involved in HSV-1 infection. The applicants have further shown that neither SB203580 [25] a specific inhibitor of p38 kinase, or Wortmannin [26] a specific inhibitor of PI3-kinase, had any measurable effect on HSV-1 infectivity.

The applicants have studied the effect of three ras effector mutant cell lines V12C40, V12G37 and V12S35 [27-29] on the ability of ras to increase the infectivity of NIH-3T3 cells to HSV-1. All three cell lines have a common activating G12V mutation as well as one other unique mutation in *Ras*, causing them to activate distinct pathways downstream of *Ras*. Mutant V12G37 is unable to signal via the RAF/ERK and the PI3-kinase pathway, but allows signaling via the RAL-GDS pathway. The V12C40 mutation disrupts signaling via the

RAF/ERK and the RAL-GDS pathways, but does not affect the PI3-kinase pathway. The V12S35 mutant cannot signal through the RAL-GDS and the PI3-kinase pathway, but can do so via the RAF/ERK pathway. PDCR represents NIH-3T3 cells which have been transfected with control vector and exert no Ras overactivity.

Our results show that the V12S35 mutant is considerably more permissive to HSV-1 infection than the other two mutants, suggesting a more significant role of the RAF/ERK pathway (as compared with the PI3-kinase or the RAL-GDS pathway) in the infection process. The role of the RAL/GDS pathway in HSV infection deserves further attention, as G37 cells are found to be susceptible to viral strain infection (not shown).

The applicants have demonstrated that non-permissiveness to HSV-1 infection in cells is predominantly at the level of α gene translation. The viral immediate early transcript α27 accumulates to comparable levels in both non-permissive and permissive cell lines. However, the viral β- and γ-class genes, whose transcription is dependent upon the presence of α gene products, are much less abundant in non-permissive cells. The α27 gene product, ICP27, is also much less abundant in non-permissive cells than in permissive cells. Without being limited to a theory, it appears that the α–gene transcripts (for example: α27) are not translated in non-permissive cells, therefore downstream events, such as β and γ gene expression, do not occur, resulting in abortive HSV-1 infection.

The mechanism of host cell non-permissiveness to viral infection is correlated with viral-transcript induced phosphorylation of an approximately 65 kDa cellular protein, determined to be a double-stranded RNA-activated protein kinase (PKR) [11]. Phosphorylated PKR will, in turn, phosphorylate eIF-2α, a translation initiation factor that efficiently inhibits viral gene translation. Permissiveness to viral infection then, is correlated to lack of phosphorylation of PKR, which means that eIF-2α is not phosphorylated, and therefore translation of viral genes can proceed.

The applicants have discovered that permissiveness to HSV-1 infection is correlated to the lack of PKR phosphorylation in transformed cells. These cells, which are permissive to HSV-1, have a lower capability of inducing PKR/eIF2a phosphorylation following viral infection as compared with non-permissive cells. It appears that HSV-1 permissive cells either lack the ability to phosphorylate PKR, or have an enhanced ability to dephosphorylate PKR, or its downstream elements, for example eIF-2α.

Attenuated mutants of HSV-1 are being tested as anti-cancer agents in clinical trials, but the exact mechanism of their anti-cancer effect is unknown. HSV-1 mutant R3616 [2] contains deletions of both copies of the viral $\gamma_1$34.5 gene. The gene product of $\gamma_1$34.5 (called ICP34.5) presumably forms a complex with protein phosphatase 1, and redirects its activity to dephosphorylate eIF-2α [30-32]. Since PKR phosphorylates eIF-2α, ICP34.5 therefore plays an antagonistic role to PKR- *i.e.* it acts "anti-PKR", and mutant R3616 is a virus that has lost its inherent anti-PKR mechanism.

The applicants have discovered that at least one reason that these mutant HSV-1 viruses selectively kill cancer cells is that elements of the Ras pathway inactivate PKR or inhibit the phosphorylation of PKR. If inactivated, PKR is unable to phosphorylate eIF-2α, and viral infection proceeds to kill the cancer cells. In normal cells, the Ras pathway is not up-regulated and therefore anti-PKR activity is not manifested. As a result PKR is active and phosphorylates eIF-2α, which blocks translation of viral transcripts thereby inhibiting viral infection.

Therefore, HSV-1 exploits both the host cell and/or viral anti-PKR mechanism for infection (see Figure 14). When the viral intrinsic anti-PKR mechanism is weakened or destroyed, as is the case with R3616, a stronger host cell anti-PKR "arm" is required to compensate for this loss, such that productive infection can result. Host cells with an activated Ras pathway (e.g. certain cancer cells) would therefore support viral growth, whereas normal cells, whose Ras pathway activity does not normally reach the threshold level to inactivate PKR, will not be able to support viral growth. Thus, viruses whose intrinsic anti-PKR mechanism is rendered ineffective would be "attenuated", in that they are incapable of infecting normal cells, but become cytolytic in cells with a strong anti-PKR activity, as would be found in cancer cells.

Based upon these discoveries Applicants have developed methods for creating attenuated viruses or identifying wild-type viruses which may be useful for use in anti-cancer therapeutics.

Modification of Viruses to Create Attenuated Viruses for use in Anti-Cancer Therapeutics

Based upon the observations that wild-type HSV 1 can infect cells that are transformed with oncogenes; that inhibitors of the Ras signaling pathway suppress HSV-1

infection; that activation of the Ras signaling pathway dephosphorylates or prevents the phosphorylation of PKR; that HSV-1 mutants which have a reduced ability to suppress PKR function can infect PKR-deficient, but not PKR-containing cells, and that infectivity is generally associated with a decreased PKR or eIF2α phosphorylation, the Applicants have devised a method for altering viruses that have an inherent anti-PKR mechanism, such that they become suitable for use as anti-cancer agents.

The method of engineering viruses for use as anti-cancer agents is based upon the theory, which has been shown by the inventors to be true for HSV 1, that a virus which has lost the inherent anti-PKR activity attributable to $\gamma_1$34.5 will selectively infect neoplastic cells over normal cells [47]. Anti-PKR activity, which can originate from the host cell or the virus, is required for viral infection. If the viral anti-PKR activity is reduced or eliminated, the host cell must compensate with a higher level of anti-PKR activity, if viral infection is to occur. Since some neoplastic cells, (i.e. *ras*-transformed cells) have elevated anti-PKR activity, they can be infected by a mutant virus that is deficient in, or lacks entirely, anti-PKR activity. On the other hand, the level of anti-PKR activity in normal cells is not sufficient to compensate for the reduction or loss of viral anti-PKR activity in the mutant virus, and infection cannot occur. The method of engineering viruses with a diminished, eliminated or altered anti-PKR activity for use as anti-cancer agents comprises the following steps:

(A) determining whether a viral strain has inherent viral anti-PKR activity;

(B) identifying the viral gene or at least one of the viral genes responsible for the viral anti-PKR activity,

(C) altering at least one of the viral gene or genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity, relative to the viral strain, is created;

(D) testing the mutant viral strain to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to infection by the mutant viral strain than are normal cells, and

(E) selecting the mutant viral strain as the viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the mutant viral strain than are normal cells.

A variety of strategies are employed by viruses in an attempt to avoid activation of, or to inactivate, the cellular PKR system, which would lead to the inhibition of viral protein synthesis. This inherent viral anti-PKR activity has been identified for example: during poliovirus infection, where PKR itself is degraded [33]; during influenza virus infection, where the viral NS1 protein blocks the binding of dsRNA to PKR [34]; during adenovirus infection, where short dsRNA (VAI RNA) is produced which binds to PKR but does not activate it [35]; during Vaccinia virus infection, where the viral K3L protein inhibits phosphorylation of eIF-2α by competing with it for binding to PKR [36]; and during HSV-1 infection, where ICP34.5 (a viral protein) binds to phosphatase-A and redirects its activity to eIF2α [38].

Viruses that are not known to have an anti-PKR activity can be investigated for the presence of such an activity using a variety of different methods known to those in the art. In a preferred method, lysates of host cells that are infected with the virus of interest, lysates from mock-infected host cells, and lysates from host cells that are treated with interferon (IFN; up to 1000U/ml for 24 hrs) are collected at various times post infection with the virus. Host cells that are treated with interferon provide a source of PKR. The preparation of the lysates is described in [37].

Lysates of mock-infected or virus-infected cells are added to an equal amount of lysate from IFN-treated cells. In addition, an activator of PKR (for example dsRNA) is added in varying amounts to different samples of mixed lysates. The level of phosphorylation of PKR is measured in each of these samples by using *in vitro*phosphorylation and immunoprecipitation methods which are described in the Examples contained herein. If the virus has an anti-PKR mechanism, the observed level of PKR phosphorylation will be lower in virus-infected cells than mock-infected cells. If such an anti-PKR activity exists, an increase in the amount of activator may be required to induce PKR phosphorylation in the samples containing extracts from virus-infected cells as compared with mock-infected cells (this increase can be as much as 100-fold). The same procedure can be conducted for detecting eIF2α-phosphorylation, as some viral products might act at the level of eIF2α and not PKR.

As mentioned above, some viruses are already known to have an inherent anti-PKR activity, and therefore step (A) of this method would be unnecessary.

Once it is known that a virus has an anti-PKR activity, the identity of the anti-PKR element is ascertained, in accordance with step (B) of this method. In some instances, it may be desirable to know the mechanism of the anti-PKR activity, as it will aid in the identification of the responsible gene and/or protein. For instance, if the anti-PKR mechanism involves competition with PKR for double stranded RNA, then the anti-PKR activity may be purified by affinity chromatography. Whether an anti-PKR activity is the result of competition with PKR for double-stranded RNA should be ascertainable by determining whether the addition of excess double-stranded RNA to the lysates of host cells that are infected with the virus of interest, restores the PKR activity in these lysates.

Affinity chromatography using dsRNA has been described in [37]. To perform affinity chromatography, cellular lysates of virus- and mock-infected cells (as controls) are incubated with Poly (rI).Poly (rC)-Sepharose beads. The beads are then washed to remove proteins that do not specifically bind to the beads. Proteins which remain bound to the beads throughout the washes are released from the beads with elution buffer. By electrophoresing the eluted protein(s) from both virus- and mock-infected cells on an acrylamide gel, proteins that specifically bound to the Poly (rI).Poly (rC)-Sepharose beads, and which are present in the virus-infected, but not the mock-infected samples are identified. Once the viral protein which competes with PKR for dsRNA is identified, the viral gene for this protein can be identified using any one of a number of techniques, such as for instance protein sequencing followed by the design of degenerate PCR primers to use for amplification of viral DNA. Alternatively, depending upon how much of the viral DNA has been sequenced already, the protein sequencing results may lead to an immediate identification of the viral gene that codes for the protein. The entire coding sequence of the viral gene can be determined using methods known to those skilled in the art. A search in any of a number of genebanks, for example a BLAST® search in the National Center for Biotechnology nucleotide database.

The anti-PKR mechanism may be degradation of PKR, as described in [33]. To identify this type of anti-PKR activity, extracts are prepared from both virus- and mock-infected cells and tested against [^{35}S]methionine-labelled PKR, to determine whether the extracts degrade PKR. Briefly, cells are washed with ice-cold PBS and lysed in lysis buffer. As a source of radiolabeled PKR, cellular extracts are prepared from HeLa cells labeled with [^{35}S]methionine and treated with beta- and alpha-Interferon (IFN). The [^{35}S]-labeled extracts are mixed with either mock- or virus-infected cell lysates for a short period of time.

Radiolabeled PKR is then immunoprecipitated from the mixture by use of appropriate antibody and subjected to SDS-PAGE analysis to determine whether there is a difference in the level of PKR between the samples, or a difference in the amount of degredation of PKR between the samples (as observed by streaks, rather than distinct bands of protein).

In order to identify the protein that is responsible for the PKR degradation, routine protein purification techniques that are known to those skilled in the art can be used and degradation activity can be followed by assaying fractions using the *in vitro* assay for PKR-degradation. An example of the use of this technique is in [33]. For example, virus-infected cells can be lysed in cold lysis buffer, nuclei and membranes removed by centrifugation and lysates ammonium sulfate fractionated. The pellets are resuspended and, after dialysis against lysis buffer containing glycerol, assayed for their ability to degrade PKR as described above. The active fraction is then subjected to further purification procedures such as gel-exclusion and/or ion exchange chromatograpy and other techniques known to those skilled in the art. At this point, the protein responsible for the PKR degradation may be identifiable on a SDS-poylacrylamide gel. Once the protein is identified, the viral gene for the protein can be identified using any one of a number of techniques, such as for instance protein sequencing followed by the design of degenerate PCR primers to use for amplification of viral DNA. Alternatively, depending upon how much of the viral DNA has been sequenced already, the protein sequencing results may lead to an immediate identification of the viral gene. The entire coding sequence of the viral gene can be determined using methods known to those skilled in the art.

Some viruses produce a viral protein that acts as a pseudosubstrate for PKR, for example by competing with eIF-2α for phosphorylation by PKR. Examples include the tat protein of HIV and the K3L protein of vaccinia virus. Detection of such viral pseudosubstrates can be carried out using an *in vitro* kinase assay containing various combinations of dsRNA, PKR, and the (putative) viral protein. An example of how this method has been used is described in [40]. Reactions containing [γ-^{32}P]ATP and PKR purified to the mono-S stage [46] can be conducted as described [39] in the presence of dsRNA derived from viral strain (which activates PKR). Substrate competition assays containing eIF2α and the putative viral protein(s), derived from the cellular lysates of virus-infected cells, can be carried out to determine if a viral protein(s) acts as a pseudosubstrate, as

described in [40]. For example, in addition to the purified eIF2α in the reaction mix, increasing amounts of the viral proteins are added. To each tube, the kinase assay containing activated PKR (as described above) is added, reactions are incubated for a period of time, and stopped by the addition of Laemmli sample buffer. Proteins are resolved in an SDS-polyacrylamide gel and autoradiographed. The levels of phosphorylated eIF2α between the samples will be compared. If increasing the amount of viral proteins causes a decrease in eIF2α phosphorylation and a concomitant increase in the phosphorylation of another protein, a competition probably exists between these two substrates for phosphorylation by PKR. The experiment can be modified by keeping the concentration of viral protein constant while increasing the concentration of eIF2α. The psuedosubstrate can then be purified using techniques known to those skilled in the art, however the process of identifying the protein is greatly aided by knowing what size it migrates to in an SDS-polyacrylamide gel. Once the protein is identified, the viral gene for the protein can be identified using any one of a number of techniques, such as for instance protein sequencing followed by the design of degenerate PCR primers to use for amplification of viral DNA. Alternatively, depending upon how much of the viral DNA has been sequenced already, the protein sequencing results may lead to an immediate identification of the viral anti-PKR gene. The entire coding sequence of the viral anti-PKR gene can be determined using methods known to those skilled in the art.

The above describes only some of the methods used by viruses as an anti-PKR mechanism. Other anti-PKR mechanisms, including the redirection of phosphatases against eIF-2α, eIF-2α phosphorylation bypass (e.g. SV40 T antigen) and PKR dimerization inhibition (e.g., influenza virus activated p58, and HCV NS5A), can also be used for engineering oncolytic versions of previously known or novel viruses [43].

The above methods describe identification of the anti-PKR element by using its mechanism of action as a starting point. However, it is not necessary to know how the anti-PKR mechanism works in order to purify the element responsible therefor, and in some instances it may not be possible to determine this at this stage, in any event. The protein responsible for the anti-PKR activity may be identified by using routine protein purification techniques, known to those skilled in the art, and described in such manuals as Maniatis *et al,* in Molecular Cloning (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory), and Ausubel *et al.,* in Current Protocols in Molecular Biology (John Wiley and Sons, Inc.).

Throughout each step of the purification, the anti-PKR activity is tracked in the various samples and fractions by using *in vitro*phosphorylation and immunoprecipitation methods which are described in the Examples contained herein. Steps of protein purification that may prove to be useful include ammonium sulphate, urea or other salt precipitation; ion exchange, gel exclusion or affinity chromatography; electrophoresis, FPLC and the like. With each step of the purification, the protein of interest is selectively separated from the other proteins in the cellular extract of virally-infected cells, until such a point is reached where sufficiently pure protein can be recovered in order to perform protein sequencing. Once the protein sequence is obtained, the gene sequence can be determined, for example by designing degenerate PCR primers to amplify viral DNA, after which it can be cloned and sequenced. Alternatively, depending upon how much of the viral DNA has been sequenced already, the protein sequencing results may lead to an immediate identification of the viral gene. The entire coding sequence of the viral gene can be determined using methods known to those skilled in the art.

Once it is determined that a virus possesses an inherent anti-PKR activity, the viral gene(s) responsible for the anti-PKR activity could also be identified by genetic, rather than protein-based methods. This may be accomplished by screening available mutant strains of the virus, to determine whether they still have the anti-PKR activity that was identified in step (A) above, or which they were known to have. Alternatively, new viral mutant strains can be generated and screened for anti-PKR activity. For instance, if the anti-PKR activity of a virus is suspected to be the result of the activity of a particular gene, perhaps based upon homology to another known genes that have an anti-PKR activity, the viral gene of interest can be mutated using any one of a number of techniques referenced below.

It is logical to expect to find viral anti-PKR genes amongst viral genes which are "indispensable" for viral growth, and these genes therefore represent obvious targets for manipulation. However, one must view this statement with caution, as viral genes may be indispensable in one host cell, but not in another, according to the biochemical properties of these cells. For example, as Example 11 and 12 herein show, if the host cell line has low ras activity, such as MEF cells, mutant R3616 which has a mutation in the $\gamma_1$34.5 gene would be considered to be "indispensable", as the virus does not grow in these cells. However, H-ras cells are permissive to viral mutant R3616, suggesting that the $\gamma_1$34.5 gene is "dispensable". Therefore it is important to use a variety of host cell lines (including transformed cells) to

explore the oncolytic activity of mutants of indispensable genes. This approach should also be utilized for any genes that are altered according to the methods of this invention, in order to ensure that a useful viral mutant is not missed.

For the purpose of determining which gene in a virus provides the anti-PKR activity of that virus, the present invention provides methods for sequentially constructing and testing mutant viral strains for the loss of their anti-PKR activity. Mutants of viral genes can be created by a number of methods. These methods can disrupt the expression of a gene, for instance by interfering with the promoter region, or other regulatory elements. Viral genes can also be inactivated by insertion of a DNA sequence, such as an oligonucleotide or reporter gene into the viral coding region [44]. Viral genes can be altered by insertions, deletions and/or base changes [45, 46]. However, for our purpose, it will only be necessary to inactivate the anti-PKR gene. This will be accomplished by insertion of the HSV thymidine kinase (HSV-tk) gene into the putative anti-PKR gene. The experimental design consists of the following steps:

1. Construction of a cloned chimeric DNA fragment containing the HSV-tk gene flanked by sequences homologous to those flanking the putative anti-PKR gene.

2. Co-transfection of permissive cells with intact viral DNA and the fragment mentioned in step 1.

3. Selection of thymidine kinase-expressing virus from the progeny of cells transfected in step 2. This is achieved by plating the virus progeny (from transfected cells in step 2) on tk-negative cells (e.g. 143 cells) or similar cell lines depending on their permissiveness to the targeted virus. In the genome of these viruses, the anti-PKR gene will have been replaced with a copy of the tk gene which also serves as a selection marker.

While the above approach is relatively straightforward in the case of DNA viruses and retroviruses (which replicate through a DNA intermediate), it will only be applicable to those RNA viruses that can be genetically altered (e.g. poliovirus, influenza virus).

Following the production of the anti-PKR mutant, the complete sequence of the altered gene is determined to confirm that mutation of the desired gene has been accomplished.

Laboratory manuals such as Maniatis *et al.*, Molecular Cloning (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory), and Ausubel *et al.*, Current Protocols in Molecular Biology (John Wiley and Sons, Inc.), provide general methods in recombinant DNA technology needed for this and other procedures. A variety of kits are also available for isolation of DNA and RNA, for PCR, RT-PCR, transfection (calcium phosphate, lipid and polymer based methods) and other techniques that may be required. Kits are avialable from different sources, including Qiagen, Gibco-BRL, Ambion, Amersham, Stratagene, and Promega. If the target anti-PKR gene has to be mutated [see 47], a variety of mutagenesis tools are available from different sources as mentioned above.

After a viral strain with the desired mutation has been created, it is tested for loss of the anti-PKR activity as described above for step (A) of this process. If the anti-PKR activity is still present in the lysates of virus-infected cells, there are several possible explanations, which are listed here to be informative and not limiting. It is possible that the gene that was mutated is not responsible for the anti-PKR activity; that the mutation does not result in the actual inactivation of that gene; that the mutation does not result in actual elimination/attenuation of the anti-PKR activity associated with that gene; that there are other genes which contribute to the anti-PKR activity, and can compensate for the mutation created. Therefore, it may be necessary to make a different mutation in the gene that is suspected to be responsible for the anti-PKR activity, or to identify other viral genes that have a role in the anti-PKR activity of the virus, using the techniques outlined above and to mutate them as well.

It is possible that viral strains that already have a low or non-existent anti-PKR activity exist, either as wild-type viral strains or as mutant viral strains, and that these strains may be useful, with or without modification, as anti-cancer agents. These viral strains with low or non-existent anti-PKR activity could be identified using the *in vitro* phosphorylation and immunoprecipitation methods disclosed herein, by comparing the level of anti-PKR activity in the strain to, for example, the levels found in HSV-1, HSV R3616, or other viral strains that are engineered according to the methods of this invention and found to be useful as anti-cancer agents.

Once a viral mutant strain lacking, or having substantially reduced or otherwise altered anti-PKR activity has been created or identified by the above methods, it is tested for its ability to selectively infect transformed or cancer cell lines over normal cell lines.

Mutated and wild-type viral strains are tested for infectivity using normal, transformed or cancer cell lines. Transformed cell lines are cell lines that are transformed with an oncogene that is an activator of the Ras signaling pathway, such as *v-erbB, Sos,* or *H-ras* or any other oncogene that leads to activation of the Ras signaling pathway. Cancer cell lines are cell lines that were originally derived from cancer or tumor tissue. Normal (untransformed or non-cancerous) cell lines are used as controls. The cell line used depends upon the cell specificity of the particular virus in question and more than one cell line may be used to complete this analysis. For example, in the case of vaccinia virus and adenovirus, a number of human cancer cell lines, including breast cancer cell lines, colorectal cell lines and prostate cancer cell lines may be used in this analysis.

Cell lines are usually infected with an MOI of between 0.01-100 PFU/cell. Generally, a range of MOI's would be tested to determine the amount of inoculum to use for both the mutated and wild-type virus strains in both normal and transformed or cancer cell lines. Cells are grown in culture medium until cytopathic effects are observed, usually up to 4 days after infection, but potentially longer than that. The period of time needed to establish infection is dependent upon the virus, the cell line and other factors, but typically infection is established between 10-100 hours post-inoculation. Indicators of permissiveness to infection include cytopathic effects, as observed microscopically; viral protein production, as determined by [^{35}S] methionine labeling, Western blotting or immunofluorescence; and progeny virus production, as determined by plaque titration. These techniques are know to those skilled in the art or described in the Examples herein.

If the cells being tested do not demonstrate the induction of cytopathic effects, viral protein synthesis or virus output after inoculation with MOI's that are usual for that virus and cell type, and within the normal time period for infection to be established, then the cells will be considered to be non-permissive to the virus. If the cells being tested demonstrate the induction of cytopathic effects, viral protein synthesis or virus output after inoculation with MOI's that are usual for that virus and cell type, and within the normal time period for infection to be established then the cells will be considered to be permissive to the virus.

Mutant viral strains that lack an inherent anti-PKR activity, or have a reduced or otherwise altered anti-PKR activity, are likely to infect transformed or cancer cells, but not normal cells. This is because transformed or cancer cells have strong anti-PKR activity and can therefore compensate for the lack of, reduction or alteration of this activity in the viral

mutants. Wild-type viral strains may infect both normal and transformed, or cancer cell lines, since these viruses have their own anti-PKR activity which may compensate for the relatively weak anti-PKR activity in normal cells.

A viral strain lacking anti-PKR activity, or having a reduced or otherwise altered anti-PKR activity, useful as an anti-cancer agent, will be a strain that specifically replicates in, and lyses cancer cells while sparing normal cells. For example, such a strain will not infect untransformed NIH-3T3 cells. Such a strain should also induce regression of tumors implanted in immune compromised mice wile causing no major side effects. Such a strain should also result in an enhanced survival rate of mice with implanted tumors.

Once identified as preferentially infecting transformed or cancer cell lines, the anti-PKR activity of the mutant viral strain may need to be modified, in order to optimize the utility of the viral strain as an anti-cancer agent. For instance, it may be found that complete elimination of a viral anti-PKR activity is less effective than partial elimination of the viral anti-PKR activity, when considering the utility of the viral strain as an anti-cancer agent. Additional or alternative mutations of the viral anti-PKR gene or genes can be made by the techniques described above to fine-tune the level of anti-PKR activity, or the quality of the anti-PKR activity. For instance, mutations in the promoter or regulatory regions of these genes can be utilized to limit but not eliminate the level of expression of the wild-type anti-PKR gene of the virus. Alternatively, it may desirable to alter the quality (i.e. specificity; stability) of the anti-PKR activity to provide a viral strain that is a suitable anti-cancer agent.

The chosen or engineered viral strain may be used in methods for treating cancers in mammals. Representative mammals include mice, dogs, cats, sheep, goats, cows, horses, pigs, non-human primates, and humans. In a preferred embodiment, the mammal is a human. In the methods of the invention, viral strain is administered to a cancer in the individual mammal.

The cancer can be a solid cancer (e.g. sarcoma or carcinoma), or a cancerous growth affecting the hematopoietic system (a "hematopoietic cancer"; e.g., lymphoma or leukemia). A cancer is an abnormal tissue growth, generally forming a distinct mass, that grows by cellular proliferation more rapidly than normal tissue growth. Cancers show partial or total lack of structural organization and functional coordination with normal

tissue. As used herein, a "cancer", also referred to as a "tumor", is intended to encompass hematopoietic cancers as well as solid cancers.

The viral strain is typically administered in a physiologically acceptable carrier or vehicle, such as phosphate-buffered saline, to the cancer. The viral strain is administered in a manner so that it contacts the cells of the cancer or cancerous cells. The route by which the viral strain is administered, as well as the formulation, carrier or vehicle, will depend on the location as well as the type of the cancer. A wide variety of administration routes can be employed. For example, for a solid cancer that is accessible, the viral strain can be administered by injection directly to the cancer. For a hematopoietic cancer, for example, the viral strain can be administered intravenously or intravascularly. For cancers that are not easily accessible within the body, such as metastases or brain tumors, the viral strain is administered in a manner such that it can be transported systemically through the body of the mammal and thereby reach the cancer (e.g., intrathecally, intravenously or intramuscularly). Alternatively, the viral strain can be administered directly to a single solid cancer, where it then is carried systemically through the body to metastases. The viral strain can also be administered subcutaneously, intraperitoneally, topically (e.g., for melanoma), orally (e.g., for oral or esophageal cancer), rectally (e.g., for colorectal cancer), vaginally (e.g., for cervical or vaginal cancer), nasally or by inhalation spray (e.g., for lung cancer).

The viral strain is administered in an amount that is sufficient to treat the cancer (e.g., an "effective amount"). A cancer is "treated" when administration of viral strain to cells of the cancer effects oncolysis of the neoplastic cells, resulting in a reduction in size of the cancer, or in a complete elimination of the cancer. The reduction in size of the cancer, or elimination of the cancer, is generally caused by lysis of cancerous cells ("oncolysis") by the viral strain. The effective amount will be determined on an individual basis and may be based, at least in part, on consideration of the type of viral strain; the individual's size, age, gender; and the size and other characteristics of the cancer. For example, for treatment of a human, approximately 10^3 to 10^{12} plaque forming units (PFU) of viral strain can be used, depending on the type, size and number of tumors present. The viral strain can be administered in a single dose, or multiple doses (i.e., more than one dose). The multiple doses can be administered concurrently, or consecutively (e.g., over a period

of days or weeks). The viral strain can also be administered to more than one cancer in the same individual.

EXAMPLES

Cells and viruses

Parental NIH-3T3 and NIH-3T3 cells transfected with the Harvey-ras (*H-ras*) oncogene were a generous gift of Dr. Douglas Faller (Boston University School of Medicine). NIH-3T3 cells along with their *Sos*-transformed counterparts (designated TNIH#5) were a generous gift of Dr. Michael Karin (University of California, San Diego). Dr. H.J. Kung (Case Western Reserve University) kindly donated parental NIH-3T3 cells along with NIH-3T3 cells transfected with the *v-erbB* oncogene (designated THC-11). All cell lines were grown in DMEM containing 10% FBS.

The $PKR^{+/+}$ and $PKR^{-/-}$ mouse embryo fibroblasts were obtained from Dr. B.R.G. Williams (the Cleveland Clinic Foundation) and were grown in α-MEM containing 10% FBS and antibiotics, as described [21].

Wild-type HSV-1 strain F [HSV-1(F)] and mutant HSV R3616 were both gifts from B. Roizman and have been described [17, 22-24].

Immunoflourescent analysis of HSV-1 infection

NIH-3T3, TNIH#5, THC-11, and H-ras cells were grown in 8-well slide chambers (Falcon) and infected with HSV-1 at a MOI of 0.5 PFU/cell, or mock-infected by application of PBS to the cells in an identical fashion as the administration of virus to the cells. At 20 hours after infection, the cells were fixed in 100% acetone for 10 min and then left at room temperature to dry. The fixed and dried cells were then incubated with a fluorescein-labeled mouse monoclonal antibody to HSV-1 gC antigen (SyvaMicrotak from Behring) for 30 min at 37°C. The slides were then washed with distilled water, dried, mounted in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope on which a Carl Zeiss camera was mounted. The magnification for all pictures was 200X.

Western blot (Immunoblot) analysis

Infected cells were lysed with the single detergent lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethy-sulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100], normalized for the amount of total protein and subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose paper. The membrane was then washed and incubated with the primary antibody [rabbit antibody against all HSV-1 antigens (Dako, CA; 1:20,000); mouse anti-ICP27 or anti-gC antibody (Rumbaugh-Goodwin Institute, FL; 1:1,000); rabbit anti-ICP8 antibody (from Dr. Paul Olivo, Washington University, St. Louis; 1:1,000); mouse anti-PKR and mouse anti-eIF-2α antibody (Santa Cruz; 1:1,000); rabbit anti-phospho-PKR and rabbit anti-phospho-eIF-2α antibody (Biosource, CA; 1:1,000) followed by HRP-conjugated secondary antibody (1:2,000). After extensive washing, the blot was exposed to Lumigel detection solution (New England Biolabs) and subjected to autoradiography.

Uninfected H-ras cells were used to demonstrate the effects of FTI-1, PD98059 on ERK1/2 phosphorylation, SB203580 on ATF-2 phosphorylation, and Wortmannin on Akt phosphorylation. Briefly, subconfluent monolayer cultures were lysed with the recommended SDS-containing sample buffer, the lysate was subjected to SDS-PAGE and electroblotted onto nitrocellulose paper. Blots were probed with anti-ERK1/2 or anti-phospho-ERK1/2 antibodies (for samples treated with FTI-1 or PD98059), anti-ATF-2 or anti-phospho-ATF-2 (for samples treated with SB203580), and anti-Akt and anti-phospho-Akt (for samples treated with Wortmannin). The antibody kits were purchased from New England Biolabs (MA). FTI-1, PD98059, SB203580 and Wortmannin were purchased from Calbiochem (CA).

Polymerase chain reaction

Cytoplasmic RNA from infected cells was isolated using the RNeasy kit by Quiagen (CA). Briefly, at various times post-infection, monolayers of cells (approximately 1 x 10^7 cells) were lysed using the RLN buffer (50 mM Tris-Cl pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT and 1000 U/ml RNasin). After removal of the nuclei by centrifugation, the supernatant was mixed with buffer RLT and ethanol, and applied to an RNeasy spin column. The column was subsequently washed and RNA was eluted in water. Equal amounts of cytoplasmic RNA from each sample were then subjected to RT-PCR using random hexanucleotide primers (Pharmacia) and reverse transcriptase (GIBCO-BRL) according to the manufacturers' protocol. The cDNAs from the RT-PCR step was then

subjected to selective amplification of cDNAs of α27, U_L29, U_L30, $\gamma_1 34.5$, and U_L44. For α27 the primers 5'-CTGGAATCGGACAGCAGCCGG-3' [SEQ ID NO: 1] and 5'-GAGGCGCGACCACACACTGT-3' [SEQ ID NO: 2] were used, which produced the predicted 222 bp fragment. For U_L2 the primers used were 5'-GCGCCCCATGGTCGTGTT-3' [SEQ ID NO: 3] and 5'-CTCCGCCGCCGAGGTTC-3' [SEQ ID NO: 4], which produced the predicted 206 bp fragment. For U_L30, the primers 5'-ATCAACTTCGACTGGCCCTTC-3' [SEQ ID NO: 5] and 5'-CCGTACATGTCGATGTTCACC-3' [SEQ ID NO: 6] were used, which produced the predicted 180 bp fragment. For $\gamma_1 34.5$, the primers used were 5'-CTCGGAGGGCGGGACTGG-3' [SEQ ID NO: 7]and 5'-GCGGGAGGCGGGGAATAC-3' [SEQ ID NO: 8], which produced a predicted 282 bp fragment. For U_L44, the primers used were 5'-GCCGCCGCCTACTACCC-3' [SEQ ID NO: 9] and 5'-GCTGCCGCGACTGTGATG-3' [SEQ ID NO: 10], which produced a predicted 661 bp fragment. As a PCR and gel loading control, GAPDH primer 5'-CGGAGTCAACGGATTTGGTCGAT-3' [SEQ ID NO: 11] and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' [SEQ ID NO: 12] were used to amplify a predicted 306 bp GAPDH fragment. Selective amplification of the various cDNAs was performed using HotStarTaq DNA polymerase (Quiagen) in a MiniCycler PTC-150 (MJ-Research). PCR was carried out for 30 cycles, with each cycle consisting of a denaturing step for 1 min at 94°C, an annealing step for 2 min at 60°C, and a polymerization step for 2 min at 72°C. The PCR products were separated on a 1.5% agarose gel impregnated with ethidium bromide, and photographed under UV illumination with Polaroid 57 film.

EXAMPLE 1: Activators of the Ras Pathway Augment HSV-1 Infection Efficiency

NIH-3T3 cells are known to be poorly infectible with HSV-1. It was of particular interest to determine whether NIH-3T3 cells that were transformed with oncogenes which are activators of the Ras pathway were equally as non-permissive to HSV infection.

Monolayers of NIH-3T3 cells, *v-erbB*- (THC-11), *Sos*- (TNIH#5), or *H-ras*-transformed NIH-3T3 cells were exposed to HSV-1 (strain F) at a MOI of 0.5 PFU per cell. Cells were photographed 20 hours after infection in order to determine the cytopathic effects of the virus on these cells. Cells were then fixed, processed and reacted with a FITC-labeled mouse anti-HSV-1 gC antibody. As shown in Figure 1, little or no morphological change could be detected in NIH-3T3 cells, which exhibited a typically flattened, spread out

morphology with marked contact inhibition. In contrast, cells transformed with *v-erbB, Sos or H-ras* exhibited rounding or clumping, which are characteristic cytopathic effects of HSV-1 infection.

In order to determine whether viral proteins were being synthesized by the HSV-1 infected, oncogene-transformed cells, immunofluorescent microscopy of these cells was performed as described above. As shown in Figure 1, the results show that virus proteins were detected only in a very small population of NIH-3T3 cells, whereas in oncogene-transformed cell lines, pronounced viral protein synthesis was observed. Scale bar, 150 μm.

The amount of HSV-1 protein synthesis in these cell lines was also determined by Western Blot analysis. Cells were infected as described above, or mock-infected, and were harvested at 10, 22 or 36 hours after infection (or mock-infection). Western blot analysis was performed on these samples using rabbit polyclonal antibody against all HSV-1 antigens, as described above, and the results are shown in Figure 2. Lanes 13-16 show uninfected NIH-3T3, TNIH#5, H-ras, and THC-11 cells, respectively. As can be seen in Figure 2, viral proteins were not present in NIH-3T3 cells, but were abundant in the oncogene-transformed NIH-3T3 cells. As all these cell lines have identical doubling times, the observed differences in the level of viral protein synthesis were not due to intrinsic differences in growth rates or translational efficiencies for these cell lines.

Similar results to the above were obtained by metabolic labeling with [^{35}S]-methionine. Briefly, at 12 hours post infection, the medium was replaced with methionine-free medium containing 0.1 mCi/ml [^{35}S]-methionine. After further incubation for 36 hours, the cells were washed in PBS and lysed in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA. The nuclei were removed by low speed centrifugation and the supernatants stored at –70°C. Aliquots (normalized for protein content) were electrophoresed through SDS-polyacrylamide gels and autoradiographed. The autoradiographs demonstrated that viral proteins were not present in NIH-3T3 cells but were abundant in the transformed NIH-3T3 cells.

The doubling times for uninfected NIH-3T3 and uninfected transformed cells are identical and they show very similar patterns and levels of cellular protein synthesis. Therefore, the observed differences in the level of viral protein synthesis could not be due to intrinsic differences in growth rates or translational efficiencies for these cell lines. This was

further supported by the observation that NIH-3T3 cells could not be rendered more permissive even when infections were carried out at a lower cell density.

To determine whether the HSV-1 infected, oncogene-transformed NIH-3T3 cells were actually producing intact HSV-1 virus particles, HSV-1 virus yield from these cells was determined by plaque titration on Vero cells. Briefly, Vero cells were grown in 6-well multi-well plates. Different dilutions of virus, from 10^{-1} to 10^{-6} are applied to each well and the cells are incubated for about 1.5 to 2 hours. Agar-DMEM (10%) was overlaid on the cells, which were then incubated for about 2 to 3 days, or until cytopathic effects are observed. The wells were then stained with neutral red. Areas containing plaques remained clear, whereas viable cells stained dark red. As shown in Figure 3, THC-11, H-ras and TNIH#5 cells produced significant amounts of viral particles as early as 15 hours after infection, with titres between 1 x 10^5 and 1 x 10^6 PFU/ml. By 35 hours post-infection, the viral titres were between 1 x 10^7 and 1 x 10^8 pfu/ml. In contrast, the viral titres from infected NIH-3T3 cells at the 15- and 25-hr time points were below 1x10^4 PFU/ml. Figure 3 shows the HSV-1 virus yield from infected NIH-3T3, THC-11, TNIH#5 and H-ras cells using two different MOI's (Upper panel: MOI = 0.5 PFU's/cell; lower panel: MOI = 5 PFU's /cell)

This example illustrates that cell lines, once transformed with activators of the Ras pathway, become permissive to infection by HSV-1 and exhibit not only cytopathic effects from infection, but synthesize viral proteins and intact viral particles. In contrast, untransformed cell lines are non-permissive to HSV-1 infection.

EXAMPLE 2: Ras-activated cells lines as a diagnostic tool for HSV-1 infection

Figure 3 shows that as early as 15 hours after infection with HSV-1, measurable virus titres are produced from cell lines that are transformed with activators of the Ras pathway.

Figure 9, Panel "A" compares the sensitivity of detection of HSV-1 antibodies in three different cell lines. Cell line A549 (human lung carcinoma) is typically used in medical laboratories to diagnose HSV-1 infection. Cell line THC-11 is transformed with *v-erbB,* and H-ras cell lines are transformed with *H-ras.* All three cell lines were infected with HSV-1 at a MOI of 0.25-0.5. At 11, 13, 15, 17 or 21 hours post infection, cells were harvested and Western blot analysis was performed on these samples as described above. The results showed that at 11 hours post infection, both THC-11 and H-ras cell lines exhibited significant levels of HSV-1 protein synthesis, whereas cell line A549 did not exhibit detectable amounts

of protein until 17 hours post infection. The Panel "B" of Figure 9 shows that infection of H-ras cells by HSV-1 can be detected using immunofluorescence with anti-gC antibody, as early as 8 hours post-infection. Therefore, the use of transformed cell lines such as THC-11 or H-ras can significantly shorten the time for the diagnosis of herpes virus infections in clinical samples. This would be important in situations where life-threatening herpes virus infections require immediate medical attention.

EXAMPLE 3: The use of Farnesyl-Transferase Inhibitors to Reduce HSV-1 Infection Efficiency

Post-translational farnesylation of Ras is necessary for association of Ras with the plasma membrane, and is a crucial process for the initiation of downstream events, including the three distinct MAPK cascades. It was postulated that if Ras, or downstream events initiated by Ras, is in fact involved in HSV-1 infection, then farnesyl transferase inhibitors should block HSV-1 replication in oncogene-transformed cells.

H-ras transformed NIH-3T3 cells were exposed to the farnesyl transferase inhibitors FTI-1 at a final concentration 50 μM or 100 μM in the culture medium. Control cells were not exposed to inhibitor. At 22 hours post-infection the cells were harvested and Western blot analysis was performed on these samples using rabbit anti-HSV-1 antibody, as described above. Figure 4, panel "A" shows that compared to the control cells, which were not exposed to FTI-1 the production of viral proteins in cells that were exposed to FTI-1 is drastically reduced.

The bottom part of Figure 4, Panel "B" shows the results of Western blots of identical samples as described in the paragraph above, except that they were probed with mouse anti-ERK1/2 antibody [Erk1/2] or mouse anti-phosphoERK1/2 antibody [P-Erk1/2]. These results show that, as a result of exposure to FTI-1, there is a lack of activity of MEK1/2 which leads to reduced phosphorylation of ERK1/2 (ERK42/44).

To determine whether other inhibitors of farnesyl transferase would have the same effect on viral protein synthesis, three additional inhibitors were tested. *H-ras* transformed NIH-3T3 cells were exposed to the farnesyl transferase inhibitors FPTI-1, FPTI-2 and FTI-4 at a final concentration 150 μM, 150 μM or or 50 μM in the culture medium. Control cells were not exposed to inhibitor. At 22 hours post-infection the cells were harvested and Western blot analysis was performed on these samples using rabbit anti-HSV-1 antibody, as

described above. Figure 5, panel "A" shows that compared to the control cells, viral protein synthesis in cells that were exposed to FPTI inhibitors is drastically reduced.

Panel "B" of Figure 5 shows the effect of FTI-1 on HSV-1 infection, using immunofluorescence. As can be seen, addition of FTI-1 to the culture medium dramatically reduces the level of viral proteins detected and it appears that fewer cells are actually infected by HSV-1.

Figure 6 shows the effects of FTI-1 at two different concentrations, on the virus yield, as quantitated by plaque titration on Vero cells (described above). Cells were infected at an MOI of 0.5 PFU/cell and were harvested 22 hours post infection. The inhibitors were present for the entire duration of the infection. As can be seen, FTI-1 treatment of cells dramatically decreased virus yield.

EXAMPLE 4: The use of Inhibitors of the ERK Pathway to Reduce HSV-1 Infection Efficiency

A major pathway downstream of Ras that regulates cell growth is the ERK pathway [6]. Stimulation of this pathway requires the phosphorylation of ERK1/2 by the mitogen-activated extracellular signal-regulated kinase kinase MEK1/2 which itself is activated (phosphorylated) by Raf, a serine-threonine kinase downstream of Ras. To determine if MEK1/2 activity is required for HSV-1 infection, we studied the effect of the MEK1/2 inhibitor PD98059 [10, 16] on infected *H-ras*-transformed cells.

HSV-1 infected *H-ras* transformed NIH-3T3 cells were exposed to 40 μM PD98059 in the culture medium. Control cells (HSV-1 infected) were not exposed to PD98059. At 22 hours post-infection, the cells were harvested and Western blot analysis was performed on these samples using rabbit anti-HSV antibody, as described above. Figure 4, panel "A" shows that compared to the control cells, which were not exposed to PD98059, the production of viral proteins in cells that were exposed to PD98059 is reduced.

Figure 4 Panel "B" shows the results of Western blots of identical samples as described in the paragraph above, except that they were probed with mouse anti-phosphoERK1/2 antibody [P-Erk1/2] to demonstrate that, as a result of exposure to PD98059, there is a lack of phosphorylation of ERK1/2 (Erk42/44).

As seen in Figure 4, Panel "A", the p38 kinase specific inhibitor, SB203580 had no effect on HSV-1 infection when used at effective doses, suggesting that elements downstream of p38 kinase are likely not involved with HSV-1 infection. Panel "B" shows the results of Western blots of identical samples except that they were probed with mouse anti-phospho-ATF-2 antibody [P-ATF2]. These results show that, as a result of exposure to SB203580, there is a lack of phosphorylation of ATF-2.

Figure 6 shows the effects of PD98059 on the virus yield, as quantitated by plaque titration on Vero cells (described above). Cells were infected at an MOI of 0.5 PFU/cell and were harvested 22 hours post infection. The inhibitor was present for the entire duration of the infection. As can be seen, PD98059 treatment of cells dramatically decreased virus yield, equivalent to the effect of FTI-1 on viral yield.

Figure 6 also demonstrates the effects of SB203580 at two different concentrations, on the virus yield, as quantitated by plaque titration on Vero cells (described above). Cells were infected at an MOI of 0.5 PFU/cell and were harvested 22 hours post infection. The inhibitors were present for the entire duration of the infection. As can be seen, SB203580 treatment of cells does not effect virus yield

EXAMPLE 5: P-13 Kinase Pathway is Not Involved in HSV-1 Infection

H-ras cells that were infected with HSV-1 were exposed to Wortmannin at a final concentration of 200 nM for the entire duration of the infection. Cells were harvested at 22 hr. post infection, lysed and subjected to SDS PAGE followed by immunoblotting with a rabbit anti-HSV-1 antibody. As the left panel of Figure 7 shows, Wortmannin has no effect on HSV-1 infection. The bottom part of Panel "A" shows the results of Western blots of identical samples, except that they were probed with mouse anti-phospho-Akt antibody [P-Akt]. The inhibitory effect of Wortmannin at this concentration is indicated by the lack of Akt phosphorylation.

The right panel of Figure 7 shows the ability of HSV-1 to infect NIH-3T3 cells that express Ras effector domain mutants. The mutant cell lines V12C40, V12G37 and V12S35 all have a common activating G12V mutation as well as one other unique mutation in *Ras*, causing them to activate distinct pathways downstream of *Ras*. Mutant V12G37 is unable to signal via the RAF/ERK and the PI3-kinase pathway, but allows signaling via the RAL-GDS pathway. The V12C40 mutation disrupts signaling via the RAF/ERK and the RAL-GDS

pathways, but does not affect the PI3-kinase pathway. The V12S35 mutant cannot signal through the RAL-GDS and the PI3-kinase pathway, but can do so via the RAF/ERK pathway. Panel B of Figure 7 shows that the V12S35 mutant is considerably more permissive to HSV-1 infection than the other two mutants, suggesting a more significant role of the RAF/ERK pathway (as compared with the PI3-kinase or the RAL-GDS pathway) in this process.

EXAMPLE 6: Viral Transcripts are Generated but not Translated in NIH-3T3 Cells

To elucidate the role of the Ras pathway in HSV-1 infection, it was important to identify the step at which HSV-1 infection is blocked in NIH-3T3 cells. Virus binding and internalization is known to be comparable between permissive and non-permissive cells. Therefore, the transcription of viral genes was investigated. Functional protein products of the immediate early viral α genes are required for the subsequent transcription of the polypeptide groups β and γ.

The relative amounts of HSV-1 transcripts generated in HSV-1 infected NIH-3T3 cells and *H-ras*-transformed cells was compared. Cells were infected with HSV-1 at a MOI of 0.5 PFU/cell. At 2, 5, 10, 20 and 25 hours after exposure to the virus, cells were harvested and RNA was extracted from them. Equal amounts of RNA from each sample were then subjected to RT-PCR, whereby selective amplification of specific viral cDNAs (α27, U_L29, U_L30, $\gamma_1 34.5$, and U_L44) was accomplished using the methods described in the "General Methods". GAPDH, which is constitutively expressed, served as a PCR and gel loading control. Figure 8, panel "A" shows that the immediate early transcript α27 accumulated to comparable levels in the two cell lines. However, the β and γ transcripts were preferentially synthesized in the Ras-transformed cells and barely detectable, if at all, in the non-permissive NIH-3T3 cells.

Since transcription of these β and γ genes requires immediate early α gene products, the drastic reduction in their expression in NIH-3T3 cells was likely due to the inability of the α transcripts to be efficiently translated in these cells. Therefore, the level of the protein product of the α27 gene (ICP27), the U_L29 gene (ICP8) and the U_L44 gene (gC) were compared between infected NIH-3T3 and *H-ras*-transformed cells. Cells at 20 hours post infection were harvested, and subjected to Western blot analysis using mouse anti-ICP27 antibody, as described in "General Methods". Figure 8, panel "B" shows that ICP27 was present at a much lower level in NIH-3T3 cells than in *H-ras*-transformed cells, even though

the levels of α27 transcripts were comparable between the two cell lines. Therefore, it appears that the α transcripts were not efficiently translated in NIH-3T3 cells, which in turn led to the lack of progression of downstream events as evidenced by the drastically reduced (or undetectable) levels of both the β and γ transcripts. Inefficient translation of these transcripts further reduced the output of the protein products such as gC and ICP8 to undetectable levels in NIH-3T3 cells as compared to *H-ras* transformed cells.

EXAMPLE 7: PKR is Phosphorylated in HSV-1 Treated NIH-3T3 Cells, but not in HSV-1 Infected Oncogene-Transformed Cells.

Because viral transcripts were generated but not translated in NIH-3T3 cells, it was investigated whether PKR is activated (phosphorylated) in these cells. Phosphorylation of PKR leads to inhibition of translation of viral genes presumably because activated PKR phosphorylates the translation initiation factor eIF-2α, which then inhibits translation of viral transcripts. In oncogene-transformed cells, PKR phosphorylation is prevented or reversed by Ras or one of its downstream elements, allowing viral gene translation to ensue.

NIH-3T3 cells and oncogene-transformed cells were infected with HSV-1 at a MOI of 0.5 PFU/cell, and incubated for 20 hours. Control (uninfected) cells were incubated for the same length of time. At this time, the media was aspirated off and the cells were lysed in a solution of: 20 mM HEPES, pH 7.4, 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Nonidet P-40, 2 μg/ml leupeptin, and 50 μg/ml aprotinin. The nuclei were then removed by low-speed centrifugation and the supernatants were stored at -70°C until use.

Cytoplasmic extracts were normalized for total protein concentration, using the Bio-Rad protein microassay method. Each *in vitro* kinase reaction contained 20 μl of cell extract, 7.5 μl of reaction buffer (20 mM HEPES, pH 7.4, 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10% glycerol) and 7.5 μl of ATP mixture (1.0 μCi [γ-^{32}P]ATP in 7 μl of reaction buffer), and was incubated for 30 minutes at 37°C [25]. Aliquots were then either boiled in Laemmli SDS-sample buffer and used for Western Blotting with mouse monoclonal anti-PKR antibody for the detection of total PKR or immunoprecipitated with the same antibody followed by SDS-PAGE and autoradiography for the detection of ^{32}P-labelled PKR (Figure 10). The phosphorylation state of eIF-2α, the main substrate of PKR was also measured by Western blots using antibodies against total and phosphorylated forms of eIF-2α.

The results of the Western Blot showed that PKR levels were comparable in all the four cell lines, whether infected with HSV-1, or not (Figure 10, Panel "A"). However, PKR phosphorylation was seen only in infected cells, and was consistently more pronounced in NIH-3T3 cells than in the oncogene-transformed cells. PKR phosphorylation did not occur in uninfected NIH-3T3 cells, but did occur in infected NIH-3T3 cells, which suggests that it was a virus-triggered event (compare lanes 1 and 5). The differential phosphorylation of PKR between untransformed and transformed cells is consistent with the observed difference in their capacity to promote HSV-1 protein synthesis. As shown in the bottom of Figure 10, Panel "A", although the levels of eIF-2α are constant, eIF-2α phosphorylation is enhanced in NIH-3T3 cells upon HSV-1 infection, but not in transformed (HSV-1 sensitive) cells. The different phosphorylation of PKR and eIF-2α between untransformed and transformed cells is consistent with the observed difference in their capacity to promote HSV-1 protein synthesis.

If inhibition of PKR phosphorylation is due to elements of the Ras signaling pathway then FTI-1 and PD98059 (blockers of Ras plasma membrane association and MEK activity respectively) which effectively inhibit HSV-1 protein synthesis and HSV-1 virus production in transformed cells, should restore PKR phosphorylation in infected cells. Figure 10 Panel "B" shows the effect of FTI-1 and PD98059 on PKR phosphorylation in HSV-1 infected H-ras cells. H-ras cells were exposed to FTI-1 (100 μM) or PD98059 (40 μM) and the phosphorylation state of PKR was assessed using anti-PKR ("total PKR") or anti-phospho-PKR ("P-PKR) antibodies . As can be seen, PKR phosphorylation is restored by these inhibitors. The difference in phosphorylation of PKR in infected NIH-3T3 cells and in infected MEF cells is shown in the right side of Figure 10, Panel "B". As can be seen, MEF cells, which are sensitive to HSV-1, have much lower levels of phosphorylated PKR than do NIH-3T3 cells, which are relatively non-permissive to HSV-1 infection.

EXAMPLE 8: Oncogene-Transformed Cells are more Permissive to HSV-1 Mutant R3616 than are NIH-3T3 Cells

The permissiveness of oncogene-transformed NIH-3T3 cell lines to attenuated mutants of HSV-1 was assessed by Western blot analysis. NIH-3T3 cell lines, and oncogene-transformed cell lines were infected with Mutant R3616 [17, 22-24]. Cells were harvested at 20 hours after infection and subjected to Western blotting with rabbit anti-HSV-1 antibody, as described in the "General Methods". As shown in Figure 11, as evidenced by the fact that

they make substantially more viral proteins, oncogene-transformed cell lines are more permissive to R3616 than NIH-3T3 cells.

EXAMPLE 9: PKR Deletion Enhances the Permissiveness of Cells to HSV-1 Infection

Mutant R3616 contains deletions in both copies of the $\gamma_1$34.5 gene. The gene product of $\gamma_1$34.5 (called ICP34.5) presumably forms a complex with protein phosphatase 1 and redirects its activity to dephosphorylate eIF-2α. Once dephosphorylated, eIF-2α is inactivated and unable to inhibit viral transcription. The net result of ICP34.5 activity is to dephosphorylate eIF-2α, whereas the activity of phosphorylated PKR is to phosphorylate eIF-2α. Thus, ICP34.5 plays an antagonistic role to PKR - *i.e.* it acts "anti-PKR", and mutant R3616 can be regarded as a virus that has lost its inherent anti-PKR mechanism.

A direct approach to test these hypotheses, and to define the role of PKR in HSV-1 infection, is through the use of host cells that are devoid of the PKR gene. Host cells that have lost the PKR gene should be more permissive to R3616 infection than host cells that have the PKR gene. In the absence of PKR, the absence of an anti-PKR mechanism in mutant R3616 is of no consequence. Conversely, wild-type HSV-1 infectivity should not differ between host-cells that do or do not have PKR activity. Since the anti-PKR activity is working in wild-type HSV-1, the presence of PKR in the host cell is of negated by the viral anti-PKR activity.

Primary embryo fibroblasts from $PKR^{-/-}$ mice and $PKR^{+/+}$ mice were compared in terms of permissiveness to HSV-1 and R3616 infection. Cells were infected with MOI of 0.5 PFU/cell and 20 hours after infection were harvested and subjected to Western blotting with rabbit anti-HSV-1 antibody, as described in the "General Methods". Figure 12 (right two lanes) shows that wild-type HSV-1, armed with its own anti-PKR mechanism, was able to infect $PKR^{-/-}$ and $PKR^{+/+}$ mouse embryo fibroblasts equally well. In contrast, R3616 viral proteins were synthesized at a significantly higher level in the $PKR^{-/-}$ cells than in the $PKR^{+/+}$ cells (Figure 12, left two lanes).

The level of infection by R3616 in $PKR^{-/-}$ fibroblasts is equivalent to that seen in cells transformed by *Ras*, or elements of the Ras pathway. Therefore, PKR deletion enhances host cell permissiveness to HSV-1 infection in the same way as does transformation by *ras* or elements of the Ras pathway. Further evidence for the Ras-PKR connection comes from the

demonstration that FTI-1 inhibited HSV-1 infection of $PKR^{+/+}$ cells, while having no effect on $PKR^{-/-}$ cells, as shown in Figure 13. In this experiment, cells were infected with HSV-1 at a MOI of 0.5 PFU/cell, in the presence of FTI-1. Cells were harvested at 20 hr post-infection and analyzed for viral proteins as above. Viral protein synthesis was reduced by FTI-1 in only the $PKR^{+/+}$ cells.

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WHAT IS CLAIMED IS:

1. A method for engineering a virus for use as an anti-cancer agent, which method comprises the following steps:

(A) determining whether a viral strain has inherent viral anti-PKR activity;

(B) identifying the viral gene or at least one of the viral genes responsible for the viral anti-PKR activity; and

(C) altering at least one of the viral gene or genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity, relative to the viral strain, is created.

2. The method of claim 1 further comprising the steps of:

(A) testing the mutant viral strain to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to infection by the mutant viral strain than are normal cells, and

(B) selecting the mutant viral strain as the viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the mutant viral strain than are normal cells.

3. A method for engineering a viral anti-cancer agent from a viral strain having inherent viral anti-PKR activity, which method comprises the following steps:

(A) identifying the viral gene or at least one of the viral genes responsible for the viral anti-PKR activity, and

(B) altering the viral gene or genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity, relative to the viral strain, is created.

4. The method of claim 3 further comprising the steps of:

(A) testing the mutant viral strain to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to infection by the mutant viral strain than are normal cells, and

(B) selecting the mutant viral strain as the viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the mutant viral strain than are normal cells.

5. A method for engineering a viral anti-cancer agent from a viral strain having inherent anti-PKR activity for which at least one gene associated with such anti-PKR activity is known, which method comprises the step of altering the viral gene or at least one of the genes responsible for the viral anti-PKR activity, such that a mutant viral strain with reduced or eliminated anti-PKR activity is created.

6. The method of claim 5 further comprising the steps of:

(A) testing the mutant viral strain to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to infection by the mutant viral strain than are normal cells, and

(B) selecting the mutant viral strain as the viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the mutant viral strain than are normal cells.

7. A method of identifying a viral strain useful as an anti-cancer agent, comprising the steps of:

(A) determining whether the viral strain has a low or non-existent anti-PKR activity,

(B) testing the viral strain in culture, if the viral strain has a low or non-existent anti-PKR activity, to determine whether one of (a) transformed cells or (b) cancer cells, are more permissive to the viral strain than are normal cells, and

(C) identifying the viral strain as a viral anti-cancer agent if one of (a) transformed cells or (b) cancer cells, are more permissive to the viral strain than are normal cells.

8. The method of claim 7 wherein the viral strain is a wild-type strain, a genetically-engineered viral strain, or a mutant viral strain.

9. The use of any virus engineered or identified in accordance with a method of claim 1, 2, 3, 4, 5, 6, 7 or 8, in the treatment of cancer.

10. The use of any virus engineered or identified in accordance with the method of claim 1, 2, 3, 4, 5, 6, 7 or 8, in the preparation of medicament for the treatment of cancer.

11. A method of treating cancer comprising administering to a mammal in need thereof an effective amount of a virus engineered or identified in accordance with the method of claim 1, 2, 3, 4, 5, 6, 7 or 8.

Figure 1

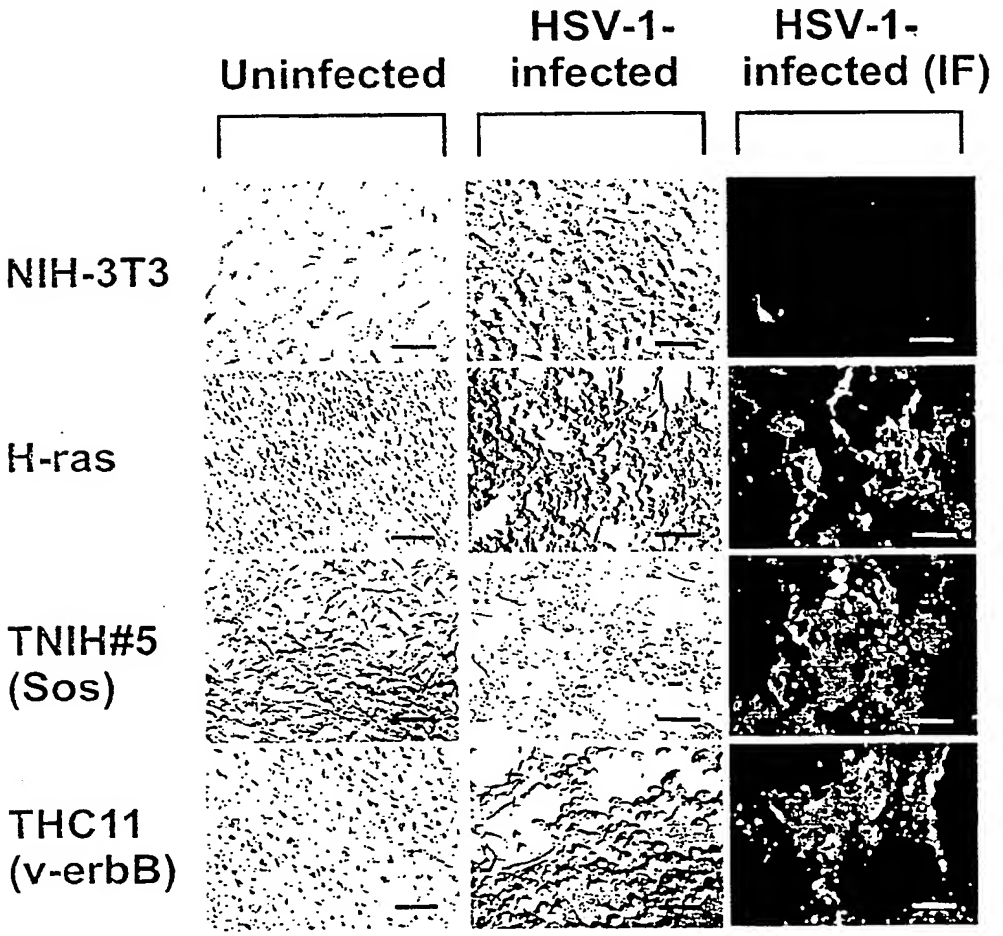
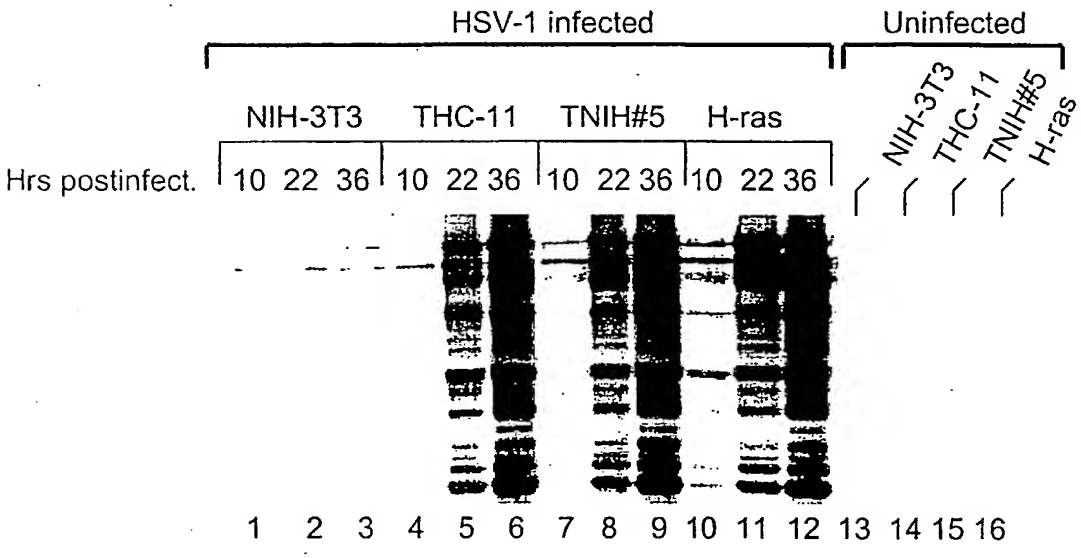


Figure 2


HSV-1 infected
Uninfected
NIH-3T3
THC-11
TNIH#5
H-ras
Hrs postinfect.
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NIH-3T3
THC-11
TNIH#5
H-ras
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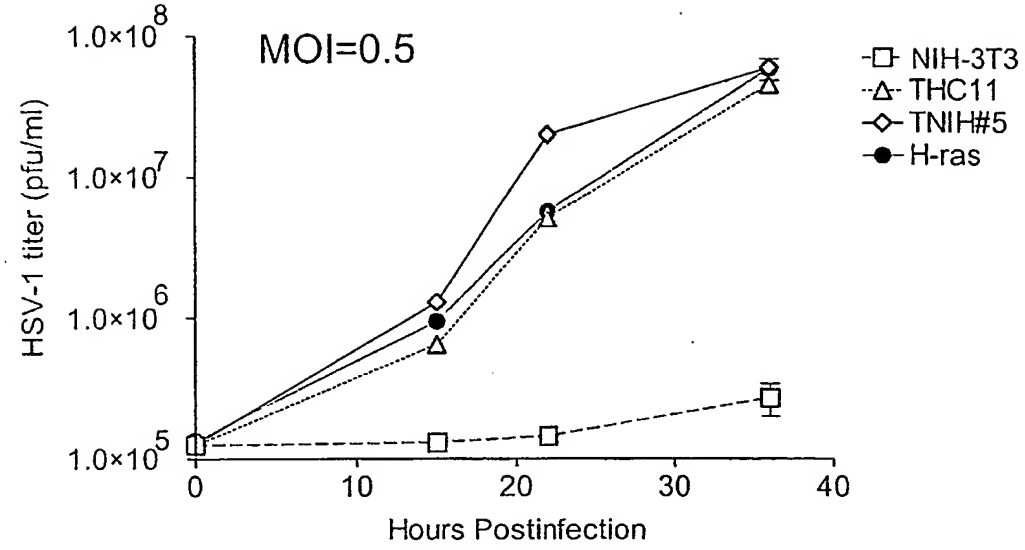
Figure 3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0
10
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30
40
Hours Postinfection
NIH-3T3
THC11
TNIH#5
H-ras

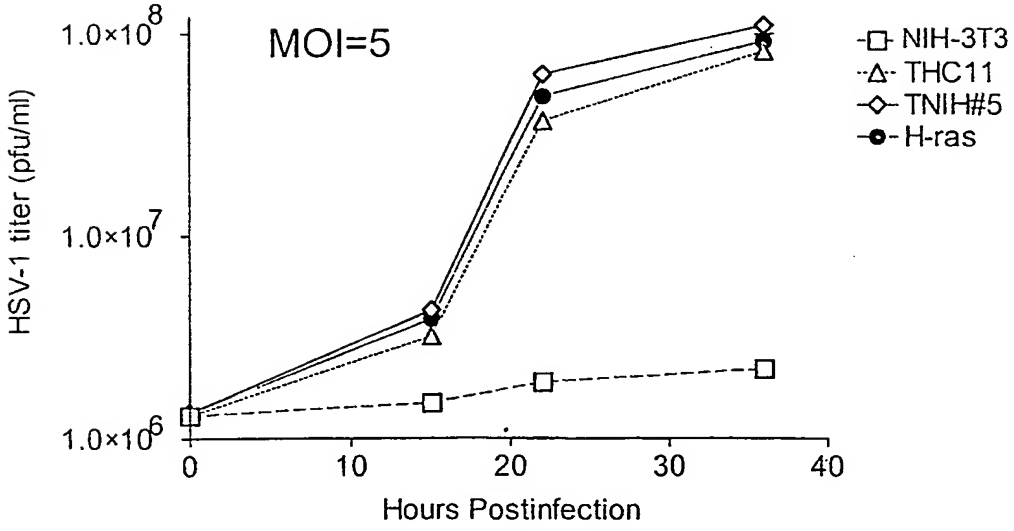

MOI=5
HSV-1 titer (pfu/ml)
1.0×10^8
1.0×10^7
1.0×10^6
0
10
20
30
40
Hours Postinfection
NIH-3T3
THC11
TNIH#5
H-ras

Figure 4

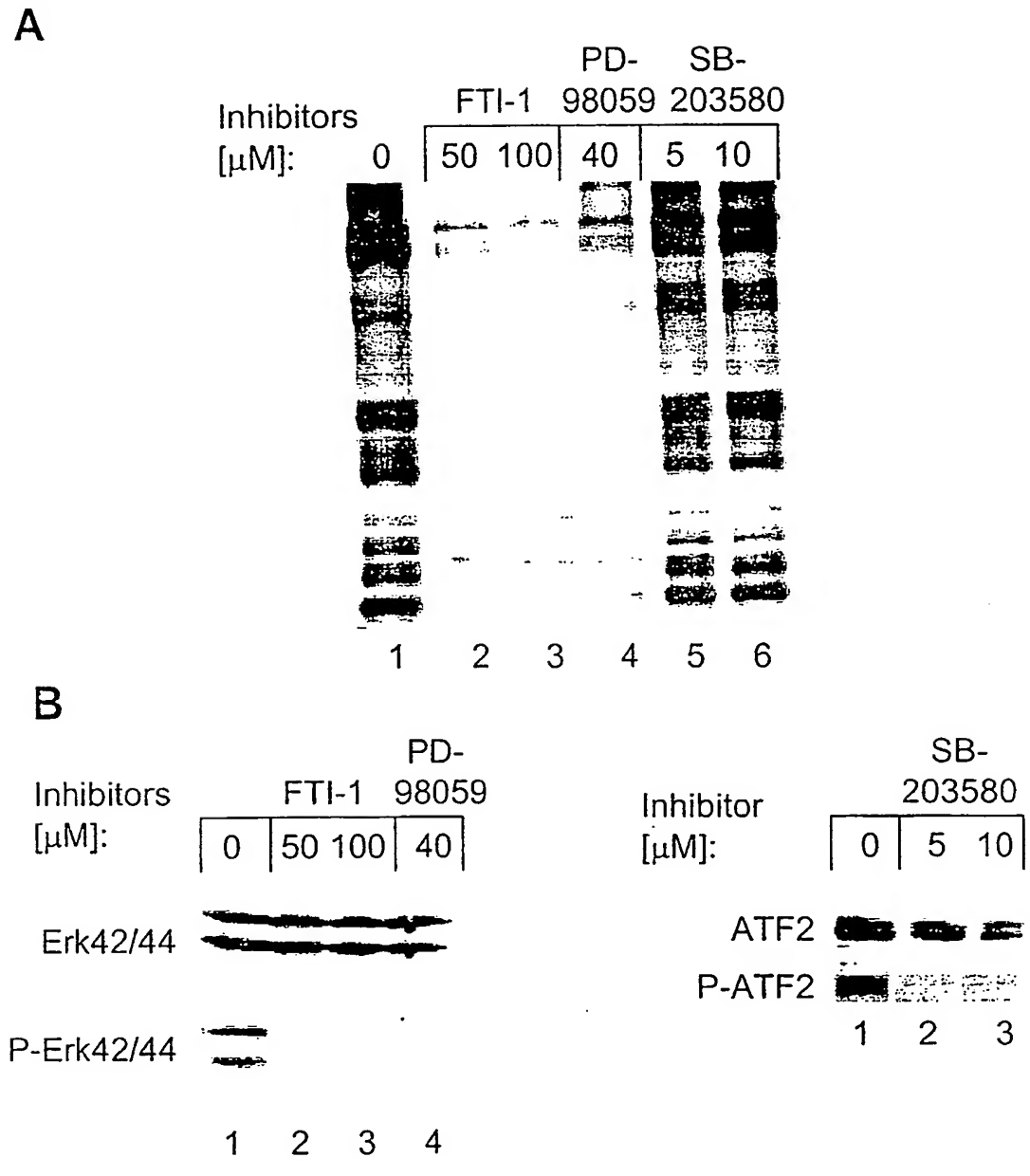
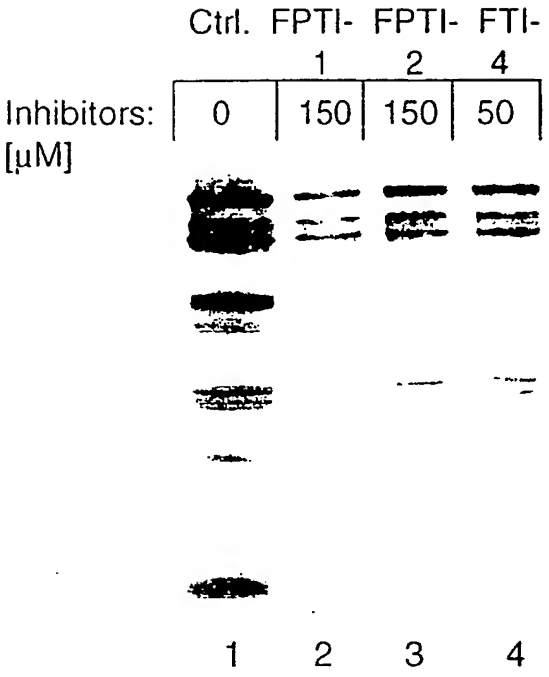

A
Inhibitors [μM]:
0
FTI-1
50 100
PD-98059
40
SB-203580
5 10
1 2 3 4 5 6
B
Inhibitors [μM]:
0
FTI-1
50 100
PD-98059
40
Erk42/44
P-Erk42/44
1 2 3 4
Inhibitor [μM]:
0
SB-203580
5 10
ATF2
P-ATF2
1 2 3

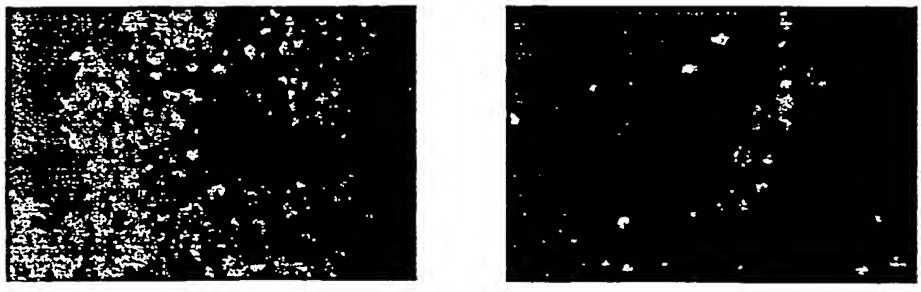
Figure 5

A



B

HSV-1-infected A549 cells



FTI-1: [100μM] – +

Figure 6

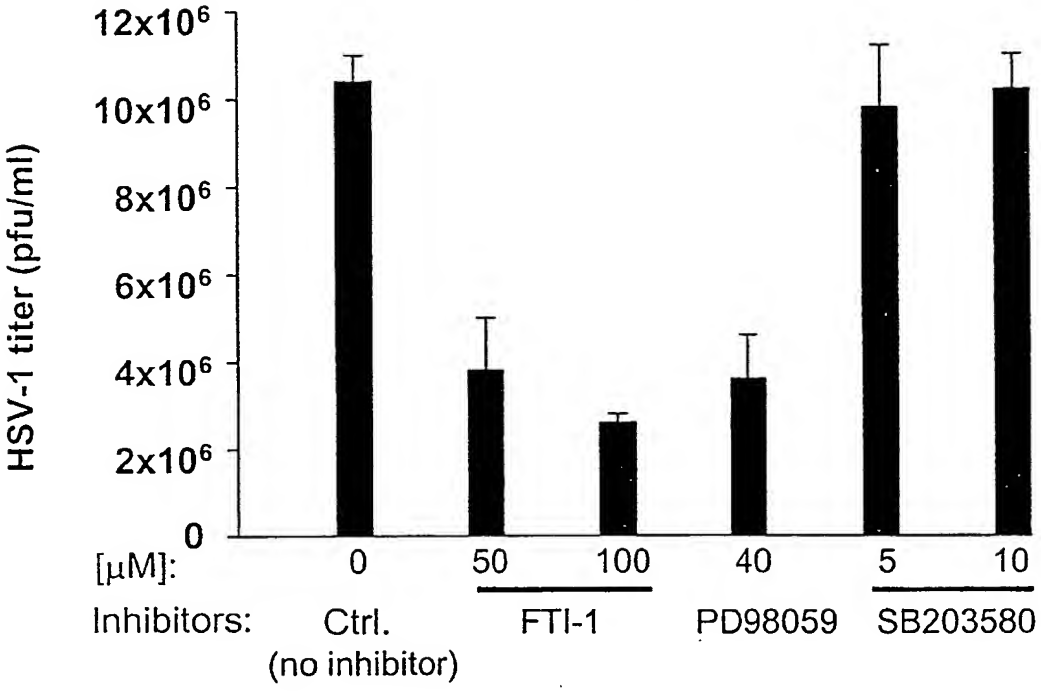

12x10^6
10x10^6
8x10^6
6x10^6
4x10^6
2x10^6
0
HSV-1 titer (pfu/ml)
[µM]:
0
50
100
40
5
10
Inhibitors:
Ctrl.
(no inhibitor)
FTI-1
PD98059
SB203580

Figure 7

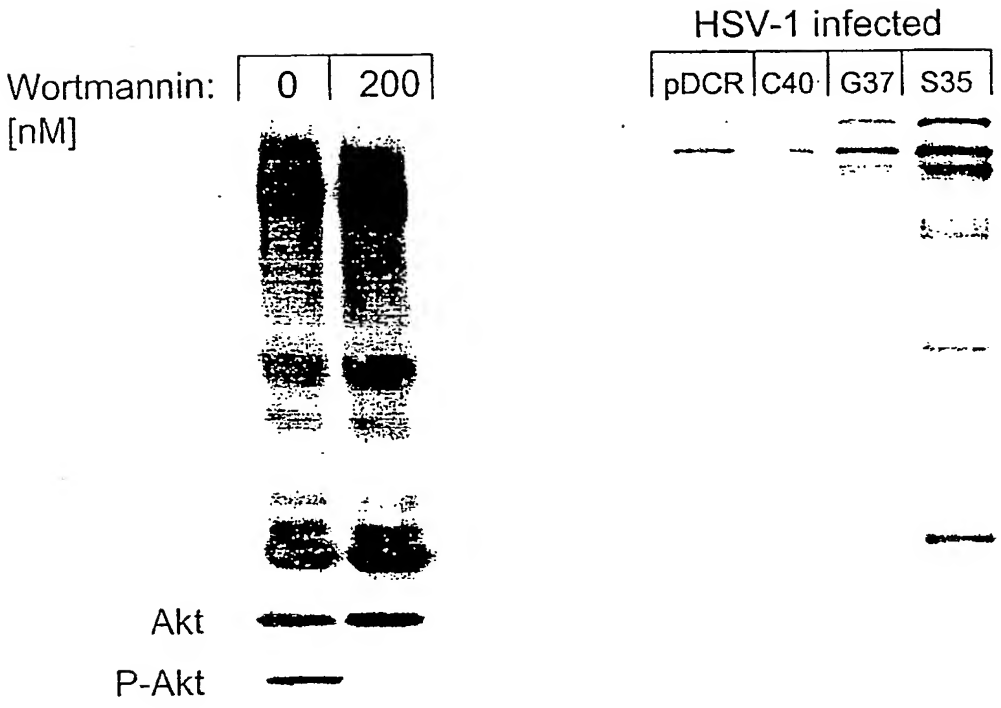
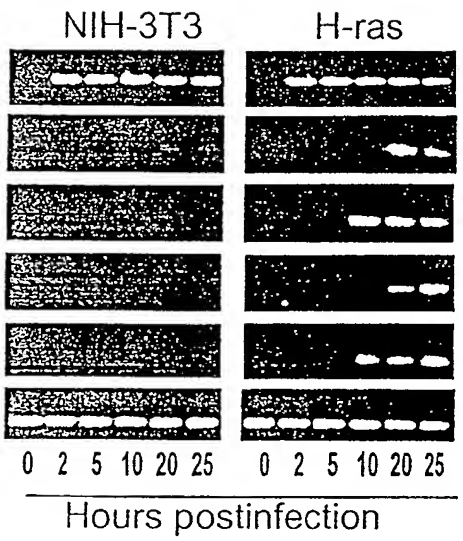


Figure 8

A



Genes	Class
α27	α
U_L29	β
U_L30	β
$\gamma_1$34.5	γ1
U_L44	γ2
GAPDH	Cellular

B

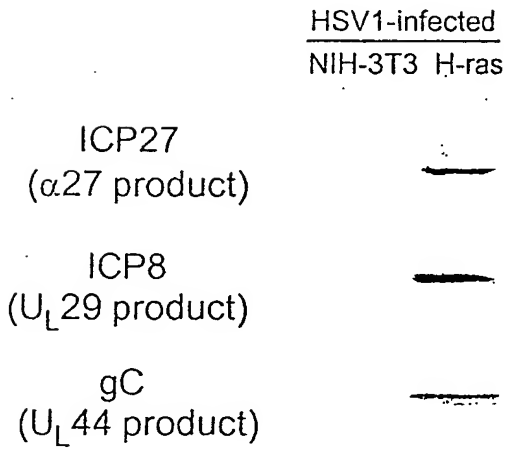
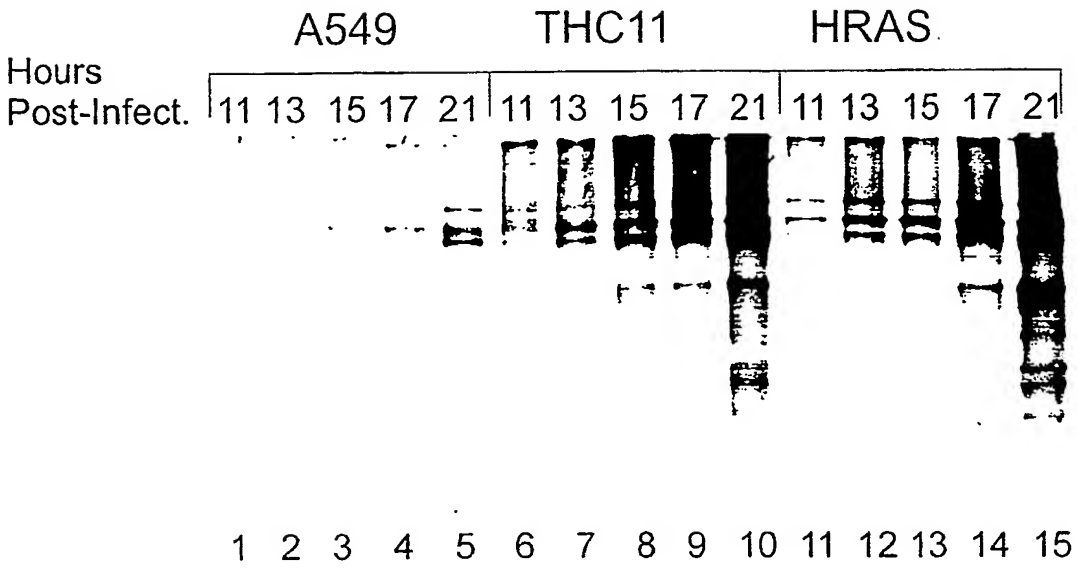


Figure 9

A

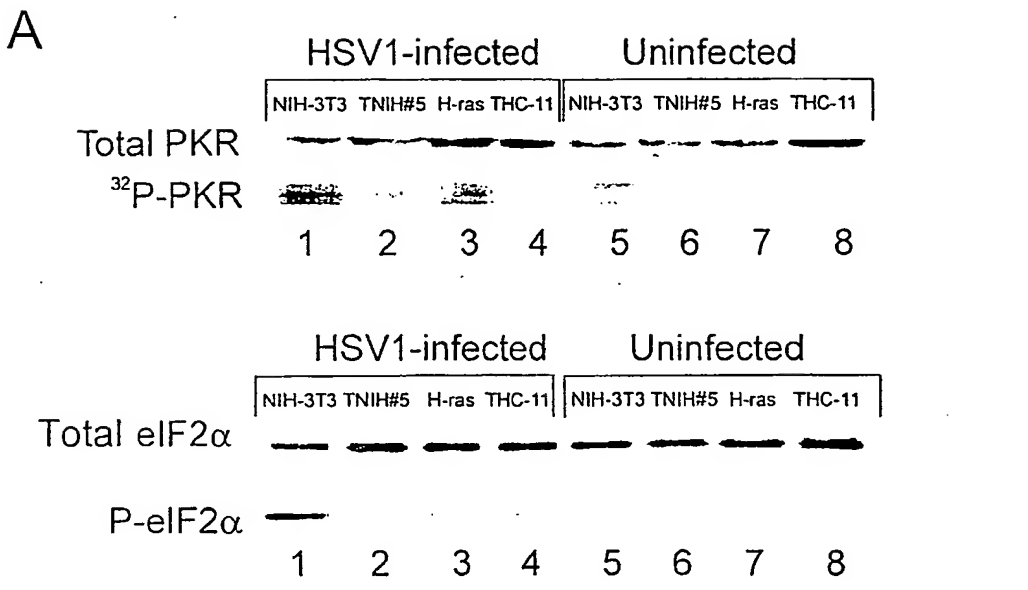


B

HSV-1-infected H-ras cells

Expression of gC protein of HSV-1 in H-ras cells as early as 8 hours post-infcetion (detected by IF).

Figure 10


A
HSV1-infected
Uninfected
NIH-3T3 TNIH#5 H-ras THC-11
NIH-3T3 TNIH#5 H-ras THC-11
Total PKR
32P-PKR
1 2 3 4 5 6 7 8
HSV1-infected
Uninfected
NIH-3T3 TNIH#5 H-ras THC-11
NIH-3T3 TNIH#5 H-ras THC-11
Total eIF2α
P-eIF2α
1 2 3 4 5 6 7 8

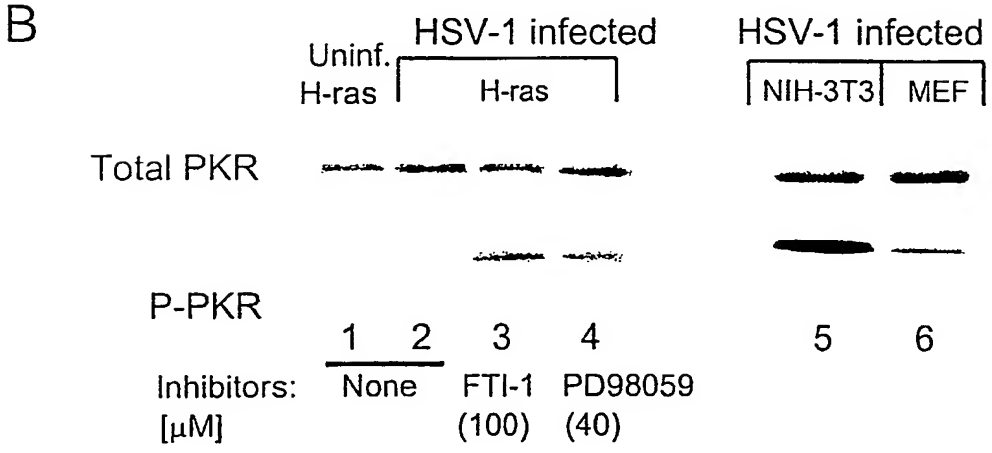

B
Uninf. H-ras
HSV-1 infected
H-ras
HSV-1 infected
NIH-3T3
MEF
Total PKR
P-PKR
1 2 3 4 5 6
Inhibitors: None FTI-1 PD98059
[μM] (100) (40)

Figure 11

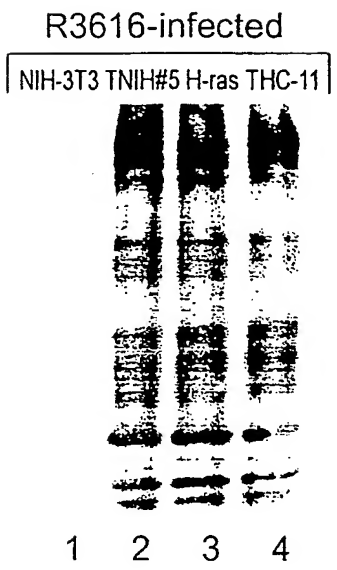


Figure 12

Figure 13

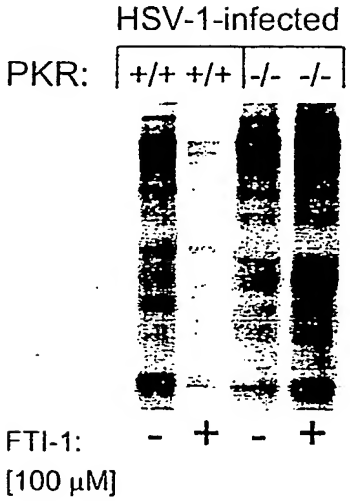
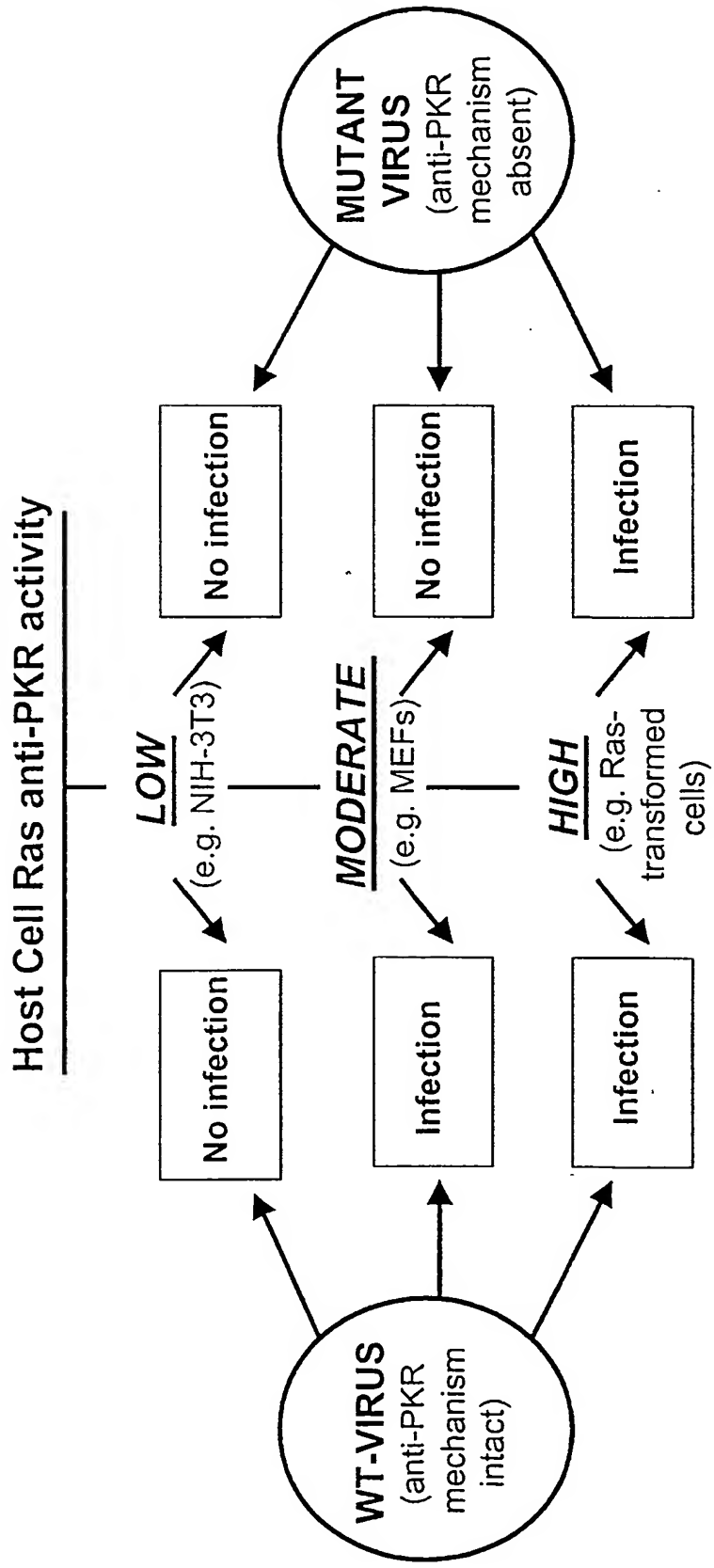


Figure 14


Host Cell Ras anti-PKR activity
LOW
(e.g. NIH-3T3)
MODERATE
(e.g. MEFs)
HIGH
(e.g. Ras-transformed cells)
MUTANT VIRUS (anti-PKR mechanism absent)
WT-VIRUS (anti-PKR mechanism intact)
No infection
No infection
Infection
No infection
Infection
Infection